Role of Mouse PinX1 in Maintaining the Characteristics of Mouse Embryonic Stem Cells

LAU, Yuen Ting

A Thesis Submitted in Partial Fulfilment

of the Requirements for the Degree of

Master of Philosophy

in

Molecular Biotechnology

The Chinese University of Hong Kong

September 2011


23 JAN 2013

Thesis/ Assessment Committee

Professor P.C. Shaw (Chair)

Professor Faye S.Y. Tsang (Thesis Supervisor)

Professor Mary M.Y. Waye (Committee Member)

Professor X.D. Li (External Examiner)

Abstract

Telomerase is crucial for maintaining telomere's length. However, its expression/activity is particularly detected in 'immortalized cells' such as cancer cells and embryonic stem cells (ESCs) whereas only little or none is found in somatic cells. It has been suggested that the high level of telomerase activity participates in maintaining the self-renewal property of ESCs by stabilizing the telomere length.

The regulation of telomerase activity is mainly on TERT, the catalytic subunit of telomerase. Currently, Pin2/TRF1-interacting protein (PinX1) was found to directly interact with TERT and regulate telomerase's activity in other cell types. We have recently showed that mouse PinX1 (mPinX1) is present in mESCs. In addition, another mPinX1 transcript variant, which is predicted to encode a novel protein isoform (named as "mPinX1t"), was detected in mESCs at mRNA level. We firstly characterized mPinX1 and mPinX1t in terms of their expression levels and sub-cellular localization in undifferentiated mESCs. By qPCR, the expression level of mPinX1 was found to be higher in undifferentiated mESCs than their differentiation derivatives, while an opposite trend was detected for mPinX1t. This hints that mPinX1 and mPinX1t may be involved in the maintenance of the undifferentiated state or differentiation processes of mESCs. By confocal microscopy, mPinX1 and mPinX1t were found to mainly localize in the nucleolus in

undifferentiated mESCs. We also confirmed the interaction of mPinX1 and mPinX1t with mouse (m)TERT by co-immunoprecipitation.

Transient knockdown of mPinX1 slightly decreased telomerase activity, while over-expression slightly increased telomerase activity, suggesting that mPinX1 might not act as a negative regulator of telomerase in mESCs as in other cell types. Both transient knockdown and over-expression of mPinX1 caused an inhibitory effect on cell proliferation without affecting cell cycle distribution and pluripotency, showing that mPinX1 might affect mESCs proliferation by other pathways. However, all the effects observed in transient experiments were not detected in stable over-expression and knockdown cell lines, probably due to some other mechanisms compensating the effect. When the stable cell lines were subjected to differentiation, mPinX1 over-expressed groups caused an advanced onset of reaching the maximum percentage of beating EBs, while mPinX1 knock-down groups caused a decreased percentage of beating EBs formed. Moreover, over-expression of mPinX1t led to a slight delay of onset of spontaneous beating in EBs. All these results hinted a potential role of mPinX1 and mPinX1t in mESC differentiation. Besides, over-expression of mPinX1t caused an increase in mPinX1 gene expression, suggesting a possible regulatory role of this splice variant on mPinX1. Further studies are needed to explore these possibilities.

摘要

端粒酶的活動對於維持端粒長度非常重要，但端粒酶的表達／活動只局限於「不死」的細胞種類，如癌細胞和胚胎幹細胞，而在普通身體細胞幾乎沒有。高端粒酶的活動能穩定端粒長度，從而令胚胎幹細胞能夠維持自我更新。控制端粒酶的活動主要決定在其催化亞單位 TERT。在其他細胞種類中，Pin2/TRF1 結合蛋白 (PinX1) 被發現能夠與 TERT 結合和控制端粒酶的活動。我們近期發現小鼠 PinX1（mPinX1）在小鼠胚胎幹細胞中表達。除此之外，我們發現了一個 PinX1 的轉錄剪接體（命名為 mPinX1t）在信使核糖核酸水平的表達，我們估計這轉錄剪接體會編碼成一新同種形蛋白質。在定量多聚酶鏈反應中，發現 mPinX1 在小鼠胚胎幹細胞的表達水平高於它的分化衍生物，相反於 mPinX1t 的表達模式。這示意 mPinX1 和 mPinX1t 可能涉及維持未分化狀態或分化程序。mPinX1 和 mPinX1t 在小鼠胚胎幹細胞中主要存在於細胞核。在免疫共沈澱實驗中，mPinX1 和 mPinX1t 均被證實與 TERT 蛋白有相互作同。在短暫提昇和降低 mPinX1 的實驗中，發現 mPinX1 在小鼠胚胎幹細胞中並不能正調整端粒酶的活動。而且 mPinX1 可能有多個控制細胞增殖的通路。可是在穩定提昇和降低實驗中卻無法偵察這些效果，估計是受到其他補償機制影響。細胞系在分化過程後，建立出來的類胚胎體出現了不同的跳動模式，示意 mPinX1 和 mPinX1t 可能涉及分化程序。除此之外，提昇表達 mPinX1t 同時亦增加了 mPinX1 基因的表達，顯示 mPinX1t 有控制 mPinX1 基因的作用，不過這些假設需要進要進一步驗證。

Acknowledgements

I would like to express my sincere gratitude to my supervisor Prof. Faye Tsang for her encouragement, guidance and helpful advices throughout the last three years.

I would also like to thank my thesis committee Prof. P.C. Shaw and Prof. Mary M.Y. Waye and Prof. X.D. Li for their suggestions and insightful comments.

Besides, my thanks are also devoted to Dr. Winnie Poon, and Ms Cecilia Leung for their encouragement, useful advices and excellent technical support.

Many thanks to my fellow labmates, especially Ms S.K. Law, Ms S.Y. Ng, Mr. C.K. Wong, Mr. Raymond Li, Mr. C.L. Tse, Ms Congie Lo, Ms C. Yang, Mr. Daniel Wu and Mr. John Li, who have given me invaluable assistances and encouragements.

Special thanks to my dearest friends in MMW610 Mr. Otto Tsang, Ms Congie Lo and Ms C. Yang who shared with me a lot of laughers and joy in these years. You guys made our lunches and teas so enjoyable, although they were always with extremely limited time. Thank you so much for cheering me up and helping me to overcome all those hard times. Our friendship is the biggest gift that I got in these two years.

Finally, I would like to devote my deepest gratitude to my close friends, family and Mr. Ivan Shek for their endless love and support in all aspects. I would like to thank my family for giving me the freedom and encouragement when I decided to choose the path of research. With all of you I would have the strength to stride across all the hurdles. I love you all.

Table of Content

List of Figures

List of Tables

List of Abbreviations

Abbreviations	Full name
APS	Ammonium persulphate
bp	Base pair
BSA	Bovine serum albumin
CaCl2	Calcium chloride
CO2	Carbon dioxide
Co-IP	Co-immunoprecipitation
Ct	Threshold cycle
DEPC	Diethyl pyrocabonate
DIG	Digoxigenin
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphoshpate
EBs	Embryoid bodies
E.coli	Escherichia coli
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ESCs	Embryonic stem cells
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green Fluorescent Protein
H3	Histone H3
HEK	Human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase
ICC	Immunocytochemistry
KCl	Potassium chloride
kDa	Kilo Dalton
LB	Luria broth
LIF	Leukemia inhibitory factor
MEF	Mouse embryonic fibroblast
mESCs	Mouse embryonic stem cells

MgSO4	Magnesium sulphate
mPinX1	Mouse Pin2/TRF1 interacting protein 1
mPinX1t	Mouse Pin2/TRF1 interacting protein 1 truncated form
mRNA	Messenger Ribonucleic acid
mTERT	Mouse telomerase reverse transcriptase
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	Molecular weight
Na	Sodium
NaCl	Sodium chloride
NaF	Sodium fluoride
NaOH	Sodium hydroxide
NCBI	Nation Center for Biotechnology Information
NEAA	Non-essential amino acids
NTC	No template control
Oct-4	Octamer-binding transcription factor 4
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline tween
PCR	Polymerase chain reaction
PFA	Para-formaldehyde
PinX1	Pin2/TRF1 interacting protein
PMSF	Phenylmethylsulfonyl fluoride
PS	Penicillin streptomycin
PVDF	Polyvinylidene difluoride
RIPA	Radio Immunoprecipitate assay
RNA	Ribonucleic acid
RNase	Ribonuclease
RQ	Relative quantification
RT	Reverse transcription
RT-PCR	Reverse transcription and routine PCR
RT-qPCR	Reverse transcription and quantitative PCR
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
shRNA	Small hairpin ribonucleic acid
Sox-2	Sex determining region Y-box 2
TAE	Tris acetate EDTA
TBP	TATA box-binding protein
TE	Tris EDTA
TEMED	N, N', N'-tetramethlenediamine

TMB	3,3',5,5'-Tetramethylbenzidine
TR	Telomerase RNA
TRAP	Telomere Repeat Amplification Protocol
UV	Ultraviolet

CHAPTER ONE INTRODUCTION

1.1 Embryonic stem cells (ESCs)

1.1.1 What are ESCs and the characteristics of ESCs

Embryonic stem cells (ESCs) are derived from the inner cell mass of early embryos called blastocyts. They were first isolated from mouse embryos in 1981 [32, 59]. ESCs have three distinct properties. Firstly, they are unspecialized, meaning that they do not have any tissue-specific structures to let them perform specialized functions. For example, they cannot co-ordinate with their neighboring cells to contract and pump blood like heart muscle cells or secrete any digestive enzymes like the secretory cells in the stomach. Secondly, they are pluripotent, which allows them to differentiate into any types of cells in the three germs layers: endoderm, mesoderm, and ectoderm. Thirdly, ESCs can undergo long term self-renewal. They can proliferate indefinitely in culture while maintaining its unspecialized and pluripotent properties.

1.1.2 Promising use of ESCs in drug development and regenerative medicine

Nowadays the high-throughput screening of drugs generally rely on the use of highly proliferative immortalized cell lines because they are easy to grow, have unlimited supply and are inexpensive. However, they may not accurately represent how the

normal human tissues response to the drug candidates [30]. ESCs is an attractive candidate for drug screening as they can proliferate in an unlimited manner and be differentiated into nearly all specific cell types, thus supplying a large variety of cells for investigating tissue specific responses of the drug candidates. (Fig.1.1)

On the other hand, ESCs could potentially provide an unlimited supply of different tissues for human transplantation [67], including those with limited regeneration capacity. Transplantation of derivatives from ESCs to replace or repair the cell lost has been proposed as future therapies for degenerative diseases, like Parkinson's disease, Huntington's disease, type I diabetes mellitus, and myocardial infarction [7, 38, 55].

Before putting ESCs into therapeutic use, some obstacles have to be overcome. A central challenge is our lack of full understanding of the intrinsic mechanism that regulates the stem cell's characteristics, and how it is regulated to become a specific type of cells [49]. The use of ESCs in transplantation is limited by its multilineage differentiaion, as the case of driving differentiation towards a single cell type using specific cultural conditions or growth factors is very rare [67]. In addition, there may be a risk of tumor formation if unwanted ESC derivatives or undifferentiated ESCs are transplanted into the recipient's body. Moreover, it is possible for immunorejection to occur, similar to the case of organ transplantation [12]. Much

more studies and researches are needed before putting ESCs into therapeutic use.

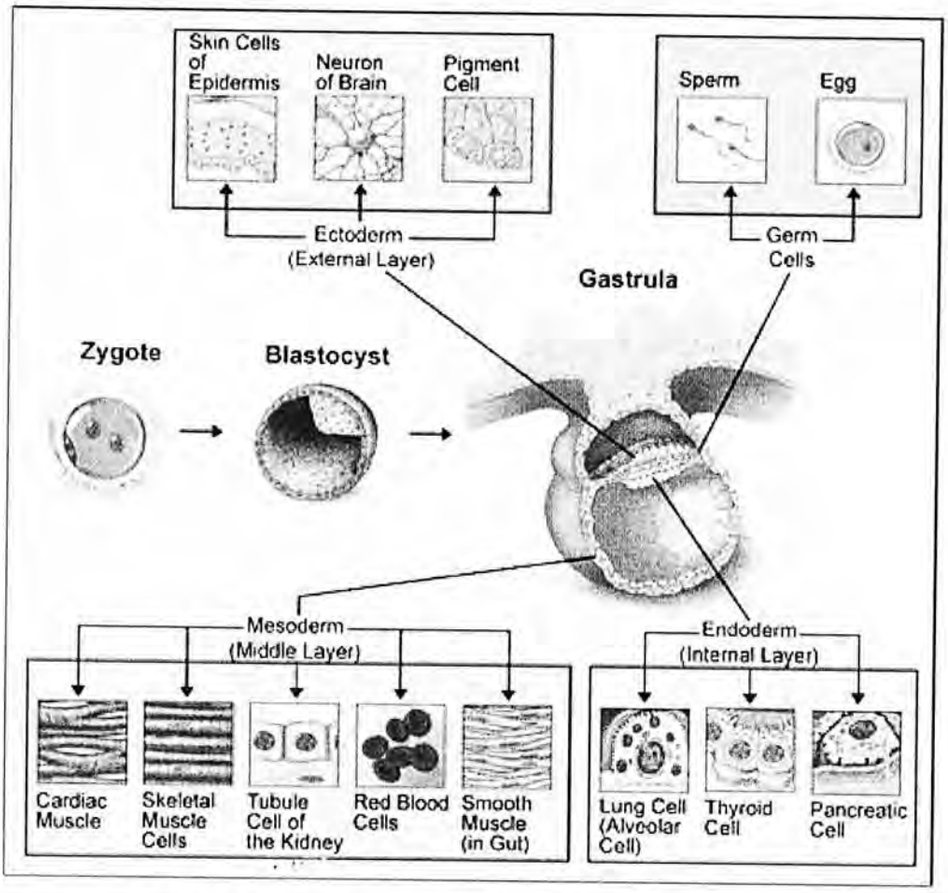


Figure 1.1 Organs derived from each germ layer, adopted from (http://www.ncbi.nlm.nih.gov/About/primer/genetics_cell.html)

1.1.3 Maintenance of self-renewal and pluripotent properties of ESCs

Both self-renewal and pluripotency are maintained in ESCs by the interactions of numerous factors, with a transcriptional network at the center [75], including Oct-4, Sox-2, Nanog, Tcf3 and the myc family. These key transcription factors auto- or cross- regulate each other so as to directly or indirectly determine the state for maintaining pluripotency and self-renewal by activating the associated genes and repressing the differentiation-promoting genes. The decrease/loss of expression of these factors may indicate the loss of pluripotency or differentiation.

Typically, ESCs are cultured and maintained on a feeder layer of mouse embryonic fibroblasts (MEFs) in the medium containing fetal bovine serum (FBS) [70]. Scientists have found that conditioned media with the addition of recombinant leukemia inhibitory factor (LIF) protein can maintain mouse ESCs (mESCs) self-renewal state without the need of MEFs feeder layer [25]. It was found that LIF could activate the STAT3 pathway which is sufficient for maintaining self-renewal in the presence of FBS. Another extrinsic factor, bone morphogenetic protein 4 (BMP4) could also corporate with LIF for maintaining self-renewal in mESCs [106]. Much work have been done to solve the puzzle of how ESCs maintain their self-renewal and pluripotent characteristics, but the distinct networking pathway behind such renewal process needs to be unriddled.

In this study, the role of PinX1, a telomerase regulator, in maintaining the characteristics of mESCs would be investigated.

1.2 Cell cycle in ESCs

1.2.1 Cell cycle

The cell cycle consists of a series of events that take place in a cell leading to the duplication of genetic materials and replication of cells. The cell cycle consists of four phases. It starts with G_1 phase, at which the cell increases in size and chromosomes prepare for replication; then it comes to the S phase where the DNA replication occurs; thereafter, it comes to G_2 phase at which the cell will continue to grow; and finally it comes to the M phase where mitosis occurs. When cell division is finished, the cells enter G_1 phase again. Cells can also enter G_0 phase and stop dividing, and at which it may also start differentiation.

To ensure the correct transmission of genetic information from generation to generation, cell cycle progression is controlled by mechanisms and checkpoints that maintain the four cell cycle phases in the correct order to ensure that DNA is replicated correctly and distributed equally to each daughter cell. Studies in various eukaryotes have demonstrated that progression through the cell cycle is driven by activation and inactivation of cyclin-dependent kinases (CDKs), which trigger the transition to subsequent phases of the cycle. CDKs are small serine/threonine protein kinases that require association with a cyclin subunit for their activation [90]. CDK levels remain constant throughout the cell cycle, while cyclins are unstable proteins

that are synthesized and degraded at precise times during the cell cycle, thus generating waves of CDK activity [98]. The precise timing of cyclin accumulation is a critical factor in cell cycle progression [73]. Cyclin protein levels are regulated by transcriptional mechanisms and at the level of protein stability. Upon cyclin binding, the CDK-subunit undergoes a series of conformational changes and adopts a catalytically active form [43]. The cell cycle progression also depends on other factors like growth factors, cell-cell contact, cell density and nutrient availability [98].

1.2.2 Characteristics of cell cycle of ESCs

The special cell cycle structure that ESCs have may play an important role for maintaining their characteristics. ESCs cell cycle consists largely of S phase and a relatively short G_1 phase [83]. Since differentiated cells spend the majority of their time in G_1 phase, the short G_1 phase of ESCs can account for their rapid rate of cell proliferation, with an unusually short generation time of approximately 8–10 hours. Moreover, expressions of most cyclins in ESCs do not oscillate periodically; instead, they are present at a rather stable amount throughout the cell cycle with a significantly elevated level when compared to all other evaluated cell types [83]. The only other cases in mammalian cells in which expressions of cyclins do not oscillate

periodically are some tumor cell lines, which have a deregulated growth property [37, 87]. This deregulated cyclin level, and hence the deregulated CDK activity, may contribute to the uncontrolled growth in these cell lines. The unusually high levels of CDK activity in ESCs can account for their rapid cell proliferation rate since suppressing CDK activity reduces ESC generation times [83]. The lack of checkpoint at the G_1 phase [11], and the absence of CDK inhibitors such as the INK family may also contribute to the special properties of ESCs [83] (Fig.1.2).

With the tight relationship between cell cycle and ESCs characteristics, knowing more about the mechanism controlling the cell cycle progression of ESCs may provide new insights into how the ESCs maintain their self-renewal and pluripotent properties.

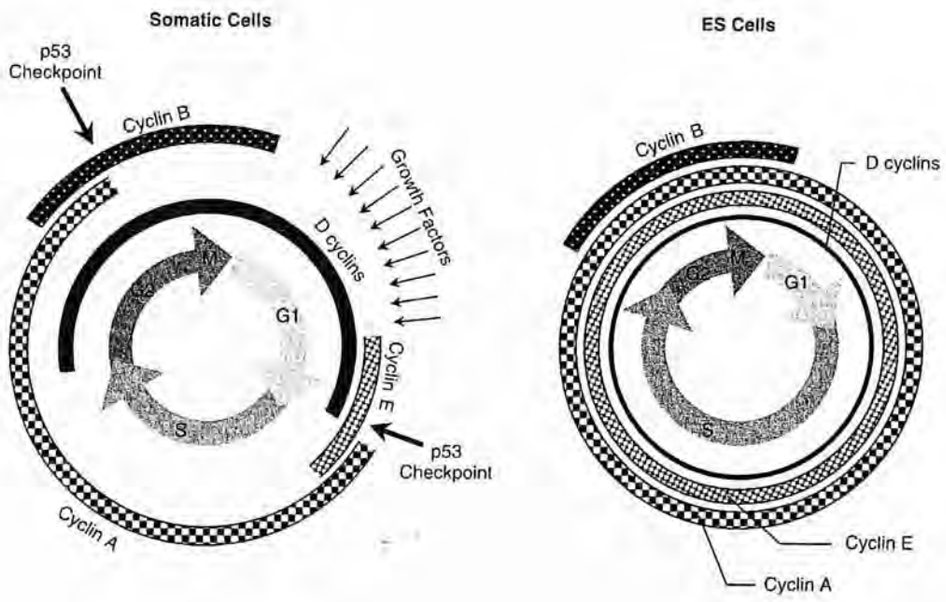


Figure 1.2 Cyclin-CDK expression patterns during cell cycle progression in somatic cells and embryonic stem cells, adapted from *Handbook of stem cells Vol: 2: Embryonic Stem Cells.*

1.3 Telomere

Telomere is the repetitive DNA sequences located at the end of linear chromosomes. In eukaryotes, this repetitive sequence is conserved with the G-rich sequence (TTAGGG)n and terminates with a 3' single-stranded overhang. The length of telomere is usually 10-15kb in human and 25-40kb in mice [4, 111, 112]. Telomere does not code for any genes but it has an important function of protecting the end of the chromosome together with the telomere-associated proteins. The telomere complex protects the DNA end from being mistaken as damaged or broken DNA and hence prevents the triggering of the DNA damage response pathway (DNA degradation and repair pathways) [14, 24, 40, 86]. Telomere is therefore very essential for maintaining the chromosome stability.

1.3.1 Telomere structure and the telomeric proteins

The telomere exists as an unusual T-loop structure in which the 3' overhang of the telomere invades the telomeric repeats of the duplex DNA and forms a displacement (D) loop. The formation of T-loop and D-loop helps to hide the 3' telomeric overhang into the duplex part, preventing the end of chromosome being recognized as damaged DNA and the activation of DNA damaged response pathway [15, 63].

Six telomeric proteins, namely POT1, TRF1, TRF2, RAP1, TIN2 and TPP1, have

been identified to be recruited in the formation of T-loop; they were also shown to be crucial for maintaining the telomeric structure and in the signaling pathways involved in telomere functions [27] (Fig.1.3). TRF1 and TRF2, each as a homodimer, bind to the double-stranded telomeric repeats. They also interact with many other proteins within the telomere signaling pathway [102]. POT1 binds to the single-stranded telomeric repeats in the D-loop for maintaining the telomere integrity [102]. TIN2 and TPP1 are crucial for the six telomeric proteins' assembly [66]. RAP1 associates with TRF2 and binds to telomere. A recent study shown that a lack of RAP1 increases telomere recombination and fragility [60]. Related to telomere functioning, TRF1, TRF2, TIN2, TPP1 and RAP1 were shown to regulate the telomere length and protect the telomere [26, 27]; TRF1, TRF2, POT1 and RAP1 were found to inhibit the DNA damage response pathway [27, 60, 79]; TPP1 was shown to be involved in telomerase recruitment [88]. Besides the telomeric roles, RAP1 has also been found to play non-telomeric roles like modulating the nuclear factor-κB (NF-κB)-mediated pathway [89].

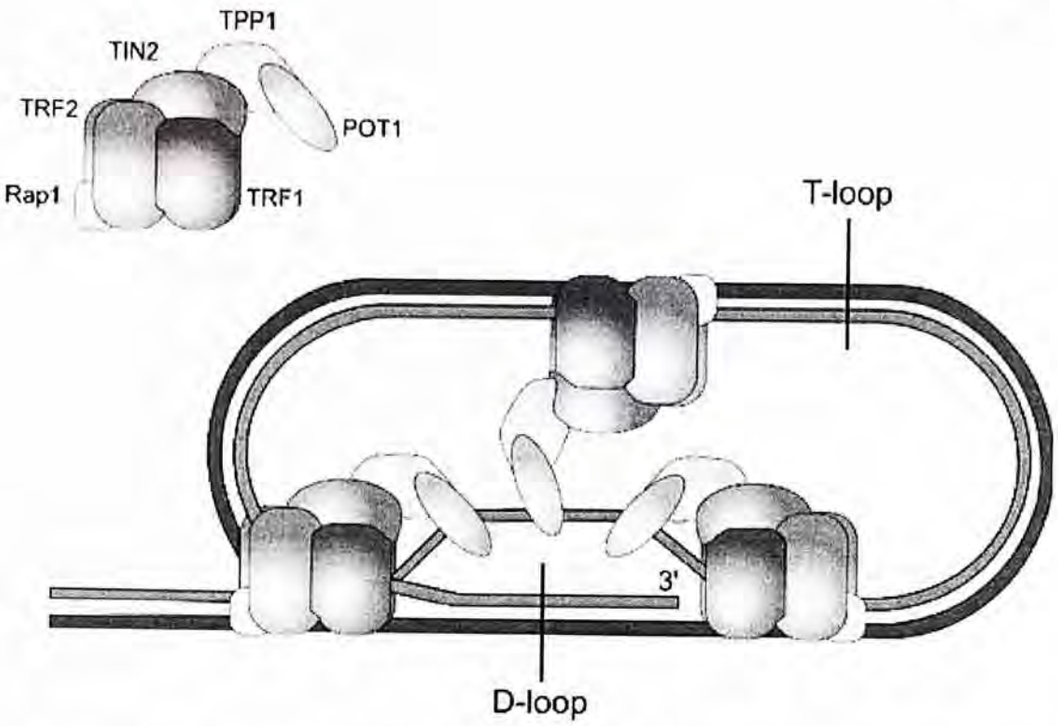


Figure 1.3 Telomere T-loop structure and the telomere associated proteins, adapted from

(http://www.springerimages.com/Images/Biomedicine/1-10.1007_s00424-009-0728-1-1)

1.3.2 End replication problem

During each cell division cycle, telomeres shorten due to the 'end replication problem'. DNA polymerase only works from 5' to 3' direction with a RNA primer at the 5' position. On the lagging strand, after the RNA primer at the 5' end is removed at the end of replication, the 5' end DNA cannot be synthesized since there is no place beyond for the primer to anneal, thus a segment at the 5' end will be lost in each round of DNA replication. This process repeats in the subsequent cell divisions and the telomere will be shortened progressively. This recurrent loss of telomere eventually leads to uncapped chromosomes and triggers the DNA damage response

pathway [26].

1.3.3 Telomere dysfunction in cancer and cellular aging

As discussed above, if the telomere becomes critically short or if there are some defects in the telomeric proteins, DNA damage response pathway will be triggered. In this case, either DNA repair, or aging, which includes the process of cellular senescence and/or apoptosis, will be elicited. When the cells try to repair the chromosome, in one case, non-homologous-end-joining pathway will be activated, causing chromosome end-to-end fusions; in another case, homologous recombination will be increased, leading to the rapid changes in telomere length and terminal deletions [28, 29]. However, chromosome instability will be resulted in both cases, leading to amplification of oncogenes and loss of tumor suppressor genes, and thus increasing the incidence of cancer. In another cellular aging pathway, the tumor suppressor p53 and/or p21 will be activated, inducing apoptosis and/or cellular senescence, and therefore stopping the cells to proliferate [17, 33].

1.4 Telomerase

Telomere length can be maintained by the action of an enzyme called telomerase. Telomerase is a ribonucleoprotein consists of the catalytic subunit, telomerase reverse transcriptase (TERT), and the template RNA (TR). By using the TR as template, TERT can add telomeric repeats onto the telomere, thereby compensating the loss of telomere during each cell cycle (Fig.1.4). By the action of telomerase, the telomere length can be maintained. Therefore, cells with high level of telomerase activity may not suffer from the problem of 'aging'. In fact, in 'immortal' cell lines such as cancer cells, embryonic stem cells and the adult stem cells, they have a detectable expression of telomerase. This telomerase activity helps maintain a stable telomere length even after many passages and gives the cells the ability to proliferate indefinitely [8, 56]. In contrast, in most somatic cells, the expression of telomerase is suppressed, limiting their proliferation capacity to 50-70 population doublings before senescence due to the critical telomere shortening [3]. Low levels of telomerase can also be detected in some highly proliferative tissues like human leukocytes, skin, mouse spleen and liver and kidney, which contain a high population of adult stem cells [20, 72].

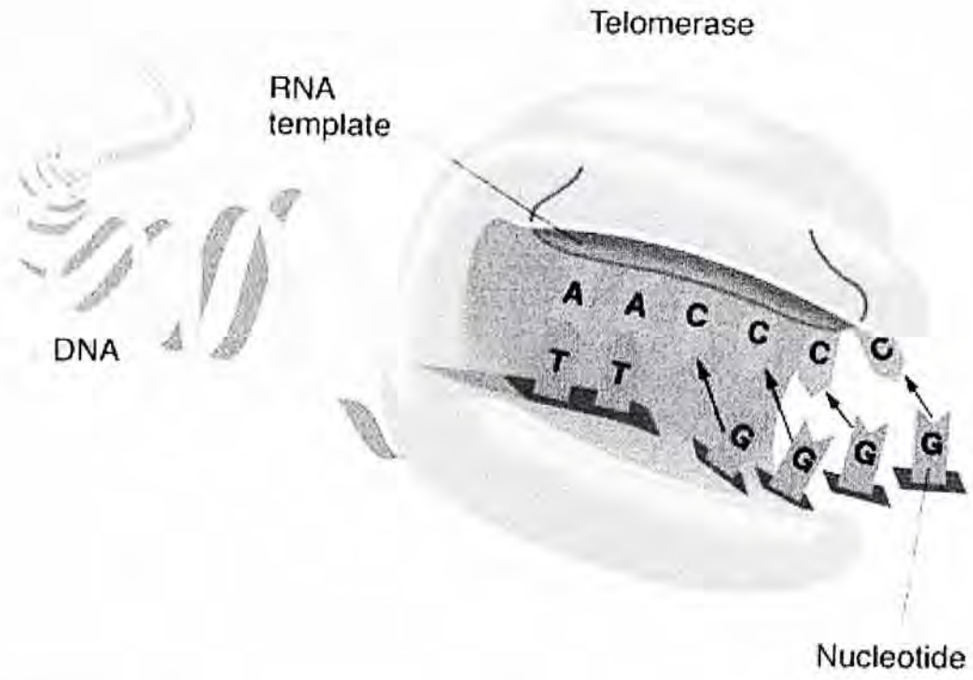


Figure 1.4 How telomerase adds new telomeric repeats onto the telomere, adapted from (http://www.martinfrost.ws/htmlfiles/sept2008/cancer-telomerase.html)

1.4.1 Telomerase and stem cell characteristics

1.4.1.1 Telomerase and cell proliferation

As mentioned in the earlier session, high telomerase expression is only restricted to immortal cells, such as cancer cells and stem cells, suggesting the importance of telomerase activity in replicative immortality. The importance of telomerase mainly comes from its ability to maintain telomere length in the cells under many rounds of cell divisions. Introduction of hTERT was done in several types of normal human somatic cells including retinal pigment epithelial cells, foreskin fibroblasts, large

vessel endothelial cells and microvascular endothelial cells [9, 39, 104]; results indicated that they had by-passed the replicative senescence and increased their maximal replication life span for more than 2 folds, in accompany with telomerase activity and maintained telomere length. Overexpression of hTERT also enhanced proliferation in hESCs and mouse skin epidermal stem cells [6, 35]. On the other hand, knock-down of TR of telomerase suppressed proliferation in HeLa cells [34, 61]. In telomerease-deficient mice, proliferation was also suppressed in skin epidermal stem cells [35]. Telomerase activity is also associated with cell cycle deregulation in many cancer types [10, 46]. All these results show that telomerase activity is positively associated with cellular proliferation in normal somatic cells, stem cells and cancer cells.

1.4.1.2 Telomerase and stem cell differentiation

Previous studies have shown that telomerase expression and activity decreased when differentiation was induced in leukemia cell lines, embryonic carcinoma cell lines and embryonic neuronal precursor cells [2, 44, 78, 80]. This might be due to the fact that differentiation causes the cells to exit from the cell cycle; since telomerase activity is positively related to the proliferation status, differentiated cells which have a lower proliferative capacity would have a lower telomerase activity. On the other

hand, mechanisms controlling differentiation may down-regulate telomerase activity.. In telomerase knockout mice, the mesenchymal stem cells was found to lose the multipotency and the ability to differentiate into chondrocytes and adipocytes [57]. Overexpression of TERT was found to change the differentiation pattern of some stem cells. Overexpression of TERT favored differentiation towards the hematopoietic lineage in mouse ESCs [6], while the same inhibited neuronal differentiation in human NT2 neural progenitor cells [74]. In contrast, a study showed that ectopic expression of TERT did not affect mouse ESCs proliferation nor differentiation, but protected ESCs against cell death during differentiation and conferred ESCs resistance to apoptosis induced by stress [48]. All these studies show that telomerase activity has an important role in the differentiation process, however, the underlying mechanism of how telomerase activity affects maintenance of undifferentiated state or differentiation process is still yet to be established.

1.4.2 Regulation of telomerase expression/ activity

1.4.2.1 Regulation of telomerase at different levels

Studies have shown that hTERT gene expression is the major determinant of telomerase activity hTERT transcript expression is low or even undetectable in most somatic cells, but it is readily detected in many of the telomerase activity positive

cells like cancer or stem cells [21, 64, 81]. The promoter region of hTERT is inactive in many somatic cells, while activated in many of the telomerase positive cells [19]. Many of the regulators of telomerase bind to the promoter region of hTERT, thereby activating or repressing the gene expression. Transcription factors c-Myc and SP1, steroid hormone estrogen and progesterone bind to the promoter of hTERT and activate its expression [45, 62, 96, 101]. On the other hand transcription factor E2F1 and Mad, tumor suppressor p53, hormone retinoic acid suppress telomerase transcription [23, 69, 71, 103].

Apart from regulation at the transcriptional level, telomerase activity can also be regulated by alternative splicing of the hTERT transcript, post-transcriptional modification, posttranslational modifications, protein-protein interactions as well as the assembly and translocation of the telomerase complex [18, 31, 100]. In this study, we would focus on a potent telomerase regulator which interacts with telomerase called PinX1, and its characteristics will be discussed in the later part.

1.4.2.2 Regulation of telomerase activity by cellular components in ESCs

Some highly expressed molecules found in ESCs which are essential for pluripotency and self-renewal are indeed regulators of telomerase expression. One example is c-Myc, which was found to regulate TERT positively at the transcriptional level [94, 101]. In ESCs culture, LIF is used for maintaining the stem cell characteristics by

activating the downstream STAT3 pathway. In fact, STAT3 is also a positive regulator of TERT expression [42]. All these studies suggest that those molecules that are highly expressed in ESCs and are essential for stem cell characteristics may also be important regulators for telomerase, thereby preventing cellular aging.

1.5 PinX1

The full name of PinX1 is Pin2/TRF1-interacting protein. PinX1 was first discovered as a Pin2/TRF1-interacting protein in a human HeLa cell yeast two-hybrid cDNA library using Pin2 as a bait by Zhou *et al.* in 2001 [112]. They firstly detected the 45kDa PinX1 protein in HeLa cells using specific antibodies. The mouse PinX1 cDNA was also cloned by PCR later by the same group [112]. Mouse PinX1 was found to encode a protein with ~74% identical to the human one. PinX1 ORF was also found in other eukaryotic cells including yeast and C. elegans, with ~50% similarity to the human one, hinting the conservation of this protein among different species [112]. Human PinX1 was found to be a potent telomerase inhibitor; it inhibited telomerase activity, shorten telomere length and promoted cellular crisis in a telomerase positive fibrosarcoma cell line [112]. Since then, PinX1 has been widely studied for its function in telomerase regulation in a variety of cancer cells as up-regulated telomerase activity accounts for cancer cell immortality.

1.5.1 Expression of PinX1

PinX1 was found to be ubiquitously expressed in various normal tissues in different species [51, 52, 68, 84]. Interestingly, hPinX1 expression decreased in a variety of human cancers [41, 51]. A previous study showed that PinX1 and telomerase

expressions were temporally regulated during *Xenopus* embryonic development: mRNA of both genes were expressed robustly during early embryogenesis and their expression decreased upon further development [91]. However, whether PinX1 is expressed at the very early stage of mammalian development at protein level and whether its expression changes upon differentiation are unknown.

1.5.2 Effects of PinX1 on the activities and the sub-cellular localization of telomerase

Distinct from other proteins that modulate telomere length without affecting telomerase activity *per se*, PinX1 was found to directly bind to telomerase and inhibit telomerase activities in several species [54, 84, 112]. In addition, both hPinX1 and yeast homolog of PinX1 were found to decrease cellular telomerase activity by sequestering TERT in the nucleolus [53, 54]. However, whether these functions are conserved in mPinX1 and mPinX1t (which lacks the C-terminal of mPinX1, will be discussed later), and whether the functions would have any impact on ESC characteristics remain unknown.

1.5.3 Structure-function relationship of PinX1

N-terminal of PinX1 consists of a glycine-rich domain (G-patch) which is highly

conserved among species [51, 52, 68, 84]. G-patch is known to bind to RNA and is frequently found in RNA-processing proteins, and yeast PinX1 was found to be involved in rRNA processing and small nucleolar RNA maturation [36].

hPinX1 was found to inhibit telomerase activity through its telomerase inhibitory domain (TID) at the C-terminal (254-326 amino acids in hPinX1) [16, 112]. Interestingly, over-expression of C-terminal alone inhibited telomerase activity and decreased cell growth to a larger extent than full-length hPinX1 [112], hinting that the hPinX1 N-terminus may activate cellular telomerase activity and promote cell growth. To study the functions of N-terminal of PinX1, few groups had tried to clone the N-terminal of PinX1 and established the N-terminal over-expressing cell line [112]. However, generation of N-terminal 1-142 amino acids over-expressing cell line was not successful in one group [112], while another group successfully generated a longer N-terminal 1-289 amino acids over-expressing cell line and found that although telomere length was extended, no change in telomerase activity and cell proliferation could be detected [16]. Therefore, the difference of the mechanism between the two termini on the regulation of telomerase activity and telomere is still largely unknown.

In addition, C-terminal and N-terminal regions of hPinX1 were shown to be important for its own nucleolar localization or exhibit inhibitory effect on its own

nucleolar localization, respectively [107]. Since PinX1 was found to sequester TERT in nucleolus and led to the unavailability of TERT to telomere [53, 54], C-terminal and N-terminal of PinX1 may have differential roles in regulating telomerase activity at the cellular level.

1.5.4 Effect of PinX1 on the growth rate of normal and cancer cells

Overexpression of hPinX1 has resulted in a decreased cancer cell growth and senescence [51, 112]. Although the knocking down of hPinX1 in normal liver cells was found to enhance cell growth [51], decreasing hPinX1 expression did not lead to detectable difference in the growth rate of cancer cells [112]. On the other hand, there are also contrasting findings showing that hPinX1 knockdown led to growth inhibition in cancer cells [110]. It was suggested that silencing PinX1 affected a telomerase-dependent pathway, causing apoptosis in a DNA damage stress condition, which would in turn affect telomere length maintenance. It is unclear whether the cellular effect is mediated through the telomerase inhibitory effect of PinX1. Altogether, full characterization of PinX1 on its role in maintaining growth of cells (including ESCs) is necessary.

1.5.5 Other functions of PinX1

Besides regulation of telomerase activity, PinX1 is also involved in some other cellular processes like mitosis. It was found that PinX1 could bind to microtubule and is essential for chromosome segregation during mitosis [108]. Another study also revealed that PinX1 interacts with a chromosome periphery protein called nucleolin, and this interaction is important for a faithful chromosome congression during mitosis [50]. On the other hand, PinX1 was also found to be involved in rRNA and small nucleolar RNA maturation, in agreement with its nucleolar localization [36].

1.5.6 Mouse homolog of PinX1 and its function in mESCs

PinX1 homolog was found to be present in various species from yeast to humans [36, 52, 68, 84, 91, 112]. The mouse homolog of PinX1 was previously cloned and protein was found to be ~74% identical to hPinX1 protein [112]. By Zhou *et al.*, mouse PinX1 was found to play a role in regulating telomerase activity and maintaining chromosome stability. Both PinX1 heterozygosity and knock-down in mouse embryonic fibroblasts led to activated telomerase and chromosomal instability [111]. However, its role in mouse ESCs is still unknown. In addition, in a PCR experiment amplifying the coding region of mPinX1 gene, we have discovered a novel splice variant of mPinX1 gene in ESCs. This novel splice variant encodes a

polypeptide chain with the N-terminal but not the C-terminal of mPinX1 (we named it as 'mPinX1t' where 't' stands for the 'truncated' form of mPinX1). The possible role of this novel mPinX1t in maintaining the characteristics of ESCs is entirely unknown. Moreover, whether this mPinX1t would have any effect on telomerase is also unexplored. On the other hand, this naturally occurring mPinX1t could provide a good opportunity to understand the cellular function of mPinX1 N-terminal.

Previous studies in the field focused on the importance of PinX1 as a tumor suppressor [41, 51, 112]. The importance of PinX1 in ESCs, which have uniquely high telomerase activity, has not been explored. Since ESCs have uniquely high telomerase activity, PinX1 may play important functions in ESCs, thus it would be worth to investigate the role of PinX1 in determining ESC characteristics.

1.6 Aims of this study

Since mESCs have uniquely high telomerase activity, mPinX1, which is a potent telomerase regulator by interacting with it, may play important functions in maintaining mESCs characteristics. Besides, we have discovered a novel splice variant of mPinX1 gene, mPinX1t, in mESCs. This novel splice variant encodes a polypeptide chain with the N-terminal but not the C-terminal of mPinX1, and the possible role of this novel mPinX1t in maintaining the characteristics of mESCs is entirely unknown. Therefore, the main aims of this study were:

1. to investigate the expression pattern of mPinX1 and mPinX1t in mESCs and their differentiation derivatives, and also in adult mouse tissues.

2. to investigate the sub-cellular localization of mPinX1 and mPinX1t in mESCs.

3. to investigate the interaction between mPinX1 and mPinX1t with mTERT.

4. to over-express and knockdown mPinX1 and mPinX1t in mESCs so as to investigate their roles in maintaining mESCs characteristics; cell proliferation, cell viability, telomerase activity, cell cycle progression, pluripotency and differentiation potential were investigated

CHAPTER TWO MATERIALS AND METHODS

2.1 mESC culture and differentiation

2.1.1 Cell line

The mESC line D3 was derived from blastocysts of a 129S2/SvPas mouse. It was purchased from American Type Culture Collection (ATCC). Human embryonic kidney cell line HEK 293 FT was purchased from Invitrogen.

2.1.2 Irradiation of MEF

MEF medium was aspirated from the T75 flask. The flask surface was washed with 5 ml PBS and then aspirated. 4ml 0.05% trypsin-EDTA solution was added and the cells were incubated at 37 ^{o}C for 3 minutes to dislodge cells. 5 ml MEF medium was added to neutralize trypsin reaction. The mixture was then pooled into a 50ml falcon and centrifuged at 1000rpm, 4 ^{o}C for 5 minutes. Supernatant was removed.. Cell counting was performed after resuspension with fresh medium. Cells required to plate for use was reconstituted to 20ml cell suspension and irradiated by gamma irradiation at 7400 rads for X minutes. Irradiated cells were transferred on ice and centrifuged at 1000rpm, 4 ^{o}C for 5 minutes. Supernatant was removed and the cell pellet was resuspended in MEF medium. Cell number was counted again and cell suspension was further diluted to 1.4 X 10^{5} cells per ml. 2 ml of cell suspension were

placed on gelatin-coated 6-well dishes and incubated at 37 °C with 5% CO_2 supply overnight to allow cells to attach.

2.1.3 mESC culture

mESCs were cultured on a 6-well plate seeded with irradiated CD-1 MEF feeder layer. Spent medium was aspirated and washed by 1ml PBS. Then, 1 ml of 0.05% trypsin-EDTA was added and the plate was incubated in 37 °C incubator for 5 minutes. After the plate was transferred to the cell culture hood, 1ml medium was then added to each well to stop trypsin reaction. Cell suspension was centrifuged at 4 °C, 1000rpm for 5 minutes. Supernatant was removed, cell pellet was resuspended in mESC undifferentiated media containing DMEM supplemented with 15% FBS, 2mM L-glutamine, 0.1mM NEAA, 0.1mM β-mercaptoethanol, 1000U/ml LIF, 50U/ml penicillin and 50μg/ml streptomycin. One-tenth of cells were made up in 2.5 ml per one well of 6-well plate and seeded on fresh CD-1 irradiated MEF for passage. The plate was returned to the 37 °C incubator with 5% CO_2 supply. Cell culture media was refreshed daily for two to three days before next passage.

2.1.4 Differentiation of mESCs

mESCs were trypsinized and resuspended in undifferentiated medium as described previously. Part of the mESCs were seeded on MEF for passage, while the remaining

cells were seeded on 0.1% gelatin-coated 100mm Petri dish and incubated at 37 °C incubator for 30 minutes. This can remove MEF in cell mixture so as to increase the purity of mESCs for differentiation. After 30 minutes, cells were centrifuge at 4 °C, 1000rpm for 5 minutes. Supernatant was removed and cell pellet was resuspended in mESC differentiation medium containing DMEM supplemented with 15% FBS, 2mM L-glutamine, 0.1mM NEAA, 0.1mM β-mercaptoethanol, 50U/ml penicillin and 50μg/ml streptomycin. Cells were counted and differentiation medium was added so that the cells were resuspended at a concentration of 4 X 10^4 cells/mL. Hanging drops were made at 800 cells per 20μl hanging drop. 50 hanging drops were placed on the lid of 100 mm Petri dish carrying 20 ml PBS to prevent drying of hanging drops. Petri dish with hanging drops was incubated at 37 °C with 5% CO_2 supply for 2 days.

On differentiation day 2, embryoid bodies (EBs) developed in hanging drops were monitored under inverted microscope to ensure single ball shape EB with normal size was formed in every hanging drop. Then, EBs were washed into 100 mm bacterial grade petri dish in 10ml suspension and incubated in 37 °C incubator with 5% CO_2 supply for 5 days.

On differentiation day 7, each EB were seeded onto one well of 0.1% gelatin coated 24-well plate with 1ml differentiation medium. The EBs were allowed to attach in

37°C incubator with 5% CO_2 supply overnight. The percentage of beating EBs was counted from differentiation day 7+1 to day 7+12. EBs were collected on required days for subsequent applications.

2.1.5 Establishment and culture of feeder-free mESCs

The mESC line D3 was first cultured on a feeder layer of irradiated MEFs on gelatin-coated culture dishes. After trypsinization, the cells were transferred to a gelatin-coated dish, incubated at 37°C for 30 minutes and passed to other gelatin-coated dishes for subsequent culture. The cells were cultured for four more passages until no MEF could be observed under microscope. The feeder-free mESCs were then kept as a continuous culture, some of which were frozen and stored for future use.

2.1.6 Culture of feeder-free mESCs

Feeder-free mESCs were cultured on 0.1% gelatin coated 6-well culture plate. They were maintained in an undifferentiated state by the culture medium which consisted of DMEM supplemented with 15% FBS, 2mM L-glutamine, 0.1mM NEAA, 0.1mM β-mercaptoethanol, 1000U/mL LIF, 50U/mL penicillin and 50μg/mL streptomycin. The cells were incubated at 37°C incubator with 5% CO_2 supply. The culture

medium was changed daily.

2.2 Trypan Blue Exclusion Assay

mESCs seeded on 12-well culture plate was trypsinized and collected in 1.5ml eppendorfs. Each well of cells was resuspended by 100μl medium. 10μl of the cells were mixed with 10μl of trypan blue and 10μl of the mixture were placed into a haemocytometer for counting of the number of dead cells (with blue staining) and viable cells (without blue staining). The total number of dead and viable cells in each well was found by multiplying the counted number with the dilution factor.

2.3 Sub-cloning

2.3.1 Amplification of the insert gene by PCR

Pairs of primers were designed to flank the coding region of sequence the gene with the addition of restriction site at both 5' and 3' primer. Polymerase chain reaction (PCR) was performed in a 50μl mixture with 0.3μM each of the primers, 1X Pfx amplification buffer, 0.3mM dNTP mixture, 1mM $MgSO_4$, 1μg template DNA and 1 unit of Platinum Pfx DNA polymerase (Invitrogen). PCR reaction was performed by denaturing at 94 ^{o}C for 2 minutes, followed by 35 cycles of amplification at 94 ^{o}C for 15 seconds, 55 ^{o}C for 30 seconds, 68 ^{o}C for 1 to 3.5 minutes, with a final extension at 68 ^{o}C for 30 seconds. For mPinX1, the primers were designed according to the

mPinX1 mRNA NCBI reference sequence NM_028228.3. For mTERT, the primers were designed according to the mTERT mRNA NCBI reference sequence NM_009354.1. Suitable restriction sites were added to the primers for subsequent restriction digestions.

Construct	**Primers (Restriction site) 5' → 3'**	**Extension time(min)**	**Template used**
pTriEx4-mPinX1/ pTriEx4-mPinX1t	Forward (EcoRI) *TAAGG*GAATTC**AT**CAGCGTTCGACAAACTTGAG	1 min	Diff. Day 7+25 mESCs cDNA
	Reverse (NotI) *TAAGG*GCGGCCGCACAGTTGAGTGGTTGGAGGC		
pWPXL-mPinX1	Forward (BclI) *AGCC*TGATCAATGTCGATGCTAGCAG	1 min	pTriEx4- mPinX1
	Reverse (MluI) *GTACG*ACGCGT**AG**TCTGGAAACTTTCTTC		
pWPXL-mPinX1t	Forward (BclI) *AGCC*TGATCAATGTCGATGCTAGCAG	1 min	pTriEx4-mPinX1t
	Reverse (MluI) *GTACA*ACGCGT**AG**GTCATGCAGAACATCT		
pCMV-HA-mPinX1/ pCMV-Myc-mPinX1	Forward (EcoRI) *AGCG*GAATTC**AG**ATGTCGATGCTAGCA	1 min	pTriEx4- mPinX1
	Reverse (NotI) *ACAT*GCGGCCGCTTATCTGGAAACTTTC		
pCMV-HA-mPinX1t/ pCMV-Myc-mPinX1t	Forward (EcoRI) *AGCG*GAATTC**AG**ATGTCGATGCTAGCA	1 min	pTriEx4-mPinX1t
	Reverse (NotI) *ACAT*GCGGCCGCTTAGTCATGCAGAACA		
pCMV-HA-mTERT/ pCMV-Myc-mTERT	Forward (EcoRI) *ATCA*GAATTC**AG**ATGACCCGCGCTCCTC	3.5 mins	pcDNA-mTERT (Kindly provided by Prof. Fuyuki Ishikawa, Kyoto University)
	Reverse (NotI) *ACAT*GCGGCCGCTTAGTCCAAAATGGTC		
pWPI-Myc-mPinX1	Forward (SwaI) *GGCC*ATTTAAATATGGCATCAATGCAGA	1 min	pWPXL-mPinX1

	Reverse (PacI) *CGGC*TTAATTAATTATCTGGA AACTTTC		
pWPI-Myc-mPinX1t	Forward (SwaI) *GGCC*ATTTAAATATGGCATCAATGCAGA	1 min	pWPXL-mPinX1t
	Reverse (PacI) *CGGC*TTAATTAATTAGTCATGCAGAACA		

Table 2.1 Forward and reverse primers used for amplification of genes in sub-cloning. For each primer, a restriction site was added as indicated by the blanket; the restriction site sequence was underlined. The clamp sequence was shown in italic. The additional bases added to prevent frame shift was indicated in bold. The specific extension time and template used for each amplification was also shown in the table.

2.3.2 Purification of PCR products

PCR products were separated by electrophoresis in a 1% TAE agarose (Invitrogen) gel with 0.7 μg/ml ethidium bromide (Invitrogen). The PCR products were visualized on a 2UV™ Transilluminator and then excised out and weighted. The DNA in gel pieces were purified using QIAquick DNA Extraction Kit (Qiagen) according to manufacturer's instructions. Briefly, the excised gels were dissolved in 3 gel weight/ volumes (g/mL) of QC buffer at 55°C for 10 minutes with occasional shaking. One gel weight/ volume of isopropanol was added to the dissolved mixture. The mixture was allowed to flow through QIAquick spin column that connected to a 2 ml collection tube. The column was then subjected to centrifugation at 13,000 rpm for 1 minute. The flow through was discarded and the column was washed once with 700μl PE buffer. The column was centrifuged at 13,000 rpm for a minute and the

flow through was discarded. The column was centrifuged again to remove any residual buffer. 30µl of autoclaved double distilled water was added and incubated for 2 minutes at room temperature and eluted by centrifugation at 13,000 rpm for 1 minute. The concentration of DNA was determined by using a Nano-drop 1000 Sepctrophotometer (Thermo Scientific).

2.3.3 Restriction enzyme digestion

For double digestion, it was performed in a 20µl reaction mixture containing 1µg of PCR product/ vector, 3 units of the two restriction enzymes (New England Biolabs), 1X BSA, 1X NEB buffer and autoclaved double distilled water. The incubation time and temperature of the reaction mixture depends on the restriction enzymes used. For serial single digestions, it was first performed in a 20µl reaction mixture containing 1µg of PCR product/ vector, 10 units of the restriction enzyme (New England Biolabs), 1X BSA, 1X NEB buffer and autoclaved double distilled water, then the reaction mixture was incubated at a temperature and for a time depending on the enzyme used. The enzyme was then heat inactivated and the PCR/vector is digested with another restriction enzyme by topping up the reaction mixture to 40µl with 10 units of the restriction enzyme (New England Biolabs), 1X BSA, 1X NEB buffer and autoclaved double distilled water. The digestion products were analyzed on a 1% TAE agarose gel and the target bands were excised and purified as described in

section 2.3.2.

Construct	**Insert/Vector cut by**	**Incubation condition**
pWPXL-mPinX1	Insert: BclI, MluI	37 °C, Overnight
	Vector: BamHI, MluI	37 °C, 2 hours
pWPXL-mPinX1t	Insert: BclI, MluI	37 °C, Overnight
	Vector: BamHI, MluI	37 °C, 2 hours
pCMV-HA-mPinX1/ pCMV-Myc-mPinX1	Insert: EcoRI, NotI	37 °C, 2 hours
	Vector: EcoRI, NotI	
pCMV-HA-mPinX1t/ pCMV-Myc-mPinX1t	Insert: EcoRI, NotI	37 °C, 2 hours
	Vector: EcoRI, NotI	
pCMV-HA-mTERT/ pCMV-Myc-mTERT	Insert: EcoRI, NotI	37 °C, 2 hours
	Vector: EcoRI, NotI	
pWPI-Myc-mPinX1	Insert: SwaI, PacI	SwaI: 37 °C 1 hour, heat inactivate at 65 °C, then PacI : 25 °C 1 hour
	Vector: SwaI, PacI	
pWPI-Myc-mPinX1t	Insert: SwaI, PacI	SwaI: 37 °C 1 hour, heat inactivate at 65 °C, then PacI: 25 °C 1 hour
	Vector: SwaI, PacI	

Table 2.2 Restriction enzymes used in sub-cloning. For generation of each construct, the restriction enzymes and specific condition used were listed in the table.

2.3.4 Ligation of digested insert and vector

Ligation of the purified digested insert and vector was performed in a 30μl reaction mixture containing 50ng of vector, insert in a 3:1, 5:1 and 8:1 mole ratio to vector, 1X ligation buffer, 600 units of T4 DNA ligase (New England Biolabs) and autoclaved double distilled water. A negative control reaction was included in

parallel by replacing DNA insert with water. The reaction mixture was incubated at 16 °C overnight for ligation.

2.3.5 Transformation of ligation product into competent cells

The frozen competent cells *E. coli* strain DH5α cells were thawed on ice. 15μl ligation mixture was mixed with 100μl competent cells and incubated on ice for 20 minutes. The cells and DNA mixture was then heat shocked at 42 °C for 90 seconds followed by chilling on ice for 3 minutes. After that, 800μl LB medium was added for recovery and cells were incubated at 37 °C for 40 minutes under shaking at 250rpm. After incubation, bacterial cells were subjected to centrifugation at 13,000g for 1 minute in Eppendorf Centrifuge 5415R. The supernatant was discarded; the cell pellet was resuspended in the remaining supernatant and was spread onto LB agar plate containing ampicillin (50μg/ml). The plate was incubated at 37 °C for overnight.

2.3.6 Confirmation of positive clone by colony PCR

The single colonies were picked from the LB agar plate and were seeded onto another plate where they were assigned with a number. Then the plate was incubated at 37 °C overnight. Some of the cells from the colonies were used to perform the colony PCR to check the existence of the insert in the colony. PCR mixture was

prepared in a 25µl mixture with 1X PCR buffer (10 mM Tris-HCl (pH 9.0), 50mM KCl), 0.2mM dNTP mixture, 1.5mM $MgCl_2$, 1 unit *Taq* polymerase (Genesys. Ltd.), 0.5mM of forward and reverse primers used in the first step of amplification, and autoclaved double distilled water. The reaction was performed by denaturing at 94°C for 3 minutes, followed by 35 cycles of amplification at 94°C for 30 seconds, 56°C for 1 minute, 72°C for 1-3.5 minutes, with a final extension at 72°C for 7 minutes. The PCR products were analyzed on a 1% TAE agarose gel.

2.3.7 Small scale preparation of the recombinant plasmid DNA

Colonies were picked from the second LB agar plate and inoculated in 5ml LB medium containing 50µg/ml ampicillin and incubated at 37°C, 250rpm for 16 hours. The bacteria were harvested by centrifugation at 6,000g for 3 minutes in Eppendorf Centrifuge 5415R on the next day. The small scale preparation of bacterial plasmid DNA was done by QIAprep Miniprep Kit (Qiagen) according to manufacturer's instructions as follows. The cell pellet was resuspended in 250µl chilled P1 Buffer. Then, 250µl P2 Buffer was added to cell suspension to lyse the cells. 350µl N3 Buffer was subsequently added to neutralize the lysis buffer and cells were subjected to centrifugation at 13,000rpm for 10 minutes. The supernatant was then applied to the QIAprep spin column and centrifuged for another minute. The flow through was

discarded and the QIAprep spin column was washed once by addition of 0.75ml PE Buffer followed by centrifugation at 13,000rpm for 1 minute. An additional centrifugation at 13,000rpm for 1 minute was performed to remove any residual wash buffer. Finally, QIAprep spin column was placed in a clean 1.5ml tube and 30µl pre-warmed autoclaved double distilled water was added to the centre of the column. The column was let stood for 1 minute and DNA was then eluted by centrifugation at 13,000 rpm for 1 minute. The concentration of DNA was determined by using a Nano-drop 1000 Sepctrophotometer (Thermo Scientific).

2.3.8 Confirmation of positive clone by restriction digestion

The prepared recombinant plasmids were double digested by specific restriction enzymes to confirm the successful ligation of the insert and the vector. The digestion procedure was performed as mentioned in 2.3.3.

2.3.9 DNA sequencing of the recombinant plasmid DNA

DNA sequencing of the recombinant plasmid DNA was performed by the commercial sequencing service TechDragon Company with a vector specific forward primer and gene specific internal primers.

2.3.10 Large scale preparation of the recombinant plasmid DNA

After verification of the positive clones, 100μl of bacteria with target insert were inoculated into 200ml LB medium containing ampicillin (50μg/ml). The culture was incubated at 37°C with shaking at 250 rpm for 16 hours. Next day, the bacterial cells were harvested by centrifugation at 4400rpm at room temperature for 15 minutes. The large scale preparation of confirmed bacterial plasmid DNA was done by QIAprep Midiprep Kit (Qiagen) according to manufacturer's instructions as follows. After the supernatant was removed, the bacterial pellet was resuspended in 6 ml chilled P1 Buffer. Afterward, 6 ml of P2 Buffer was added, mixed by inverting the sealed tubes 4-6 times, and incubated for 5 minutes to lyse the cells. The resultant viscous cell lysate was then neutralized by 6 ml chilled P3 Buffer. The lysate was then poured into the barrel of the QIAfilter Cartridge and incubated at room temperature for 10 minutes. The cap from the QIAfilter outlet nozzle was then removed and a plunger was gently inserted into the QIAfilter Cartridge to filter the cell lysate into a previously QBT Buffer-equilibrated HiSpeed Tip. The clear lysate was allowed to enter the resin by gravity flow. When all lysate was flown through, the HiSpeed Tip was washed with 20 ml QC Buffer and DNA was eluted with 5 ml QF Buffer. The DNA eluted was then precipitated by mixing with 3.5ml isopropanol and incubated at room temperature for 5 minutes. The precipitated DNA was

subsequently passed through a QIAprecipitator under constant pressure for 3 times and washed with 2 ml 70% ethanol. The QIAprecipitator was then dried by pressing air through it for several times and is allowed to air dry for 15 minutes. Finally, the DNA was eluted by 1ml pre-warmed autoclaved double distilled water. The concentration of DNA was determined by using a Nano-drop 1000 Sepctrophotometer (Thermo Scientific).

2.4 Design of siRNA targeting mPinX1 and mPinX1t

The siRNA of mPinX1 and mPinX1t were designed and synthesized by the *Silencer*® Custom Designed siRNA (Ambion). The knockdown efficiency of the siRNA were tested by transfection of the siRNA into mESCs followed by qPCR. *Silencer*® Negative Control #1 siRNA (Ambion) were used as the control scrambled siRNA.

siRNA	Sequence 5' – 3'
mPinX1 siRNA (sense)	CAAGAAGCUUGCUCAGGAUTT
mPinX1t siRNA 1 (sense)	GCAUGACUAAGAGAAGAAATT
mPinX1t siRNA 2 (sense)	CUUUGCCAGAUUAAGAUUGTT

Table 2.3 List of siRNA used in transient knockdown experiments

2.5 Transient transfection

One day before transfection, 3-10 x 10^4 cells were plated on each well of 12-well

plate. On the day of transfection, medium was aspirated, cells were washed with PBS and 1ml of medium without antibiotics was added back. Plasmid DNA/ siRNA transfection was done with Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions as follows. 1.6μg of plasmid DNA/ 60pmol of siRNA were diluted in 100μl of Opti-MEM I Reduced Serum Medium. At the same time 2-4μl of lipofectamine was diluted with 100μl of Opti-MEM I Reduced Serum Medium in another tube. They were incubated at room temperature for 5 minutes. Then the diluted plasmid DNA/ siRNA and lipofectamine were combined and incubated for another 20 minutes at room temperature. After the incubation, the 200μl were added to each well containing the cells and medium drop by drop. The cells were put back into the 37°C incubator with 5% CO_2 supply. Medium was changed after 4-6 hours. Medium was changed daily and cells were collected at 24, 48 and 72hrs after transfection. The transfection could be scaled up or down according to manufacturer's instructions.

2.6 Cloning of siRNA into shRNA insert in Lentiviral Vector pLVTHM

The siRNA of mPinX1 was designed and synthesized by the *Silencer*® Custom Designed siRNA (Ambion) and the scrambled version of the siRNA was generated by siRNA Wizard V3.1 Online. The sequences were shown in Table 2.4. The

sequence of mPinX1 siRNA and the scrambled siRNA were applied for the design of shRNA insert which generated ClaI and MluI sticky ends after annealing according to the suggestion from Tronolab (http://tronolab.epfl.ch/) as shown in Table 2.5. The oligos were ordered at 50nmole scale and dissolved in autoclaved double distilled water to get a concentration of 100μM. The oligos were annealed to form double strand inserts by mixing 1000pmol of sense and antisense oligos in 1X Annealing Buffer (100mM K Acetate, 30mM HEPES at pH7.4, 2mM Mg Acetate) and autoclaved double distilled water. The annealing mixture was initially heated to 95°C for 5 minutes, then the temperature was touched down to 67°C in 28 cycles (1 minute each). The mixture was maintained at 67°C for 30 minutes and then touched down again in 42 cycles (1 minute each) to reach 20°C which complete the annealing process. 5μl of annealed oligos were then subjected to phosphorylation by T4 PNK Kinase in 1X T4 Buffer (New England Biolabs) and autoclaved double distilled water. The phosphorylation process lasted for 30 minutes followed by 10 minutes heat inactivation of the T4 PNK Kinase at 70°C. The Lentiviral Vector pLVTHM was sequentially digested with restriction enzymes ClaI and MluI for subsequent ligation. Firstly, 2μg of pLVTHM was digested by 10 units of ClaI (New England Biolabs) in 1X NEB buffer 4 and 1X BSA with autoclaved double distilled water at 37°C for 60 minutes followed by heat inactivation of ClaI at 65°C for 20 minutes. Subsequent

MluI digestion was carried out by topping up the reaction mixture to 40µl with 20 units of MluI (New England Biolabs) in 1X NEB buffer 3 and 1X BSA with autoclaved double distilled water at 37°C for 90 minutes followed by heat inactivation of MluI at 65°C for 20 minutes. The digestion product of pLVTHM was separated in 1% TAE Agarose (Invitrogen) gel. The gel was excised out with the aid of 2UV™ Transilluminator and subjected to Gel Extraction (QIAGEN) process as mentioned in section 2.3.2. 20 to 100ng of digested pLVTHM were used to ligate with 5µl of phosphorylated oligos by T4 DNA Ligase in T4 Buffer (New England Biolabs) and autoclaved double distilled water. The ligation mixture was prepared in duplicate, one tube was incubated at 4°C for 48 hours, another tube was incubated at 16°C for 48 hours. The ligated plasmid with the mPinX1 siRNA and the Scrambled mPinX1 siRNA were named as shPinX1 and shScrambled, respectively.

siRNA	Sequence 5' – 3'
mPinX1 siRNA (sense)	AAGAAGAAAGTTTCCAGATAA
Scrambled mPinX1 siRNA (sense)	GAAACAGATAATAGACAGTTA

Table 2.4 List of siRNA for cloning into pLVTHM. The mPinX1 siRNA sequence was designed by Ambion while the scrambled siRNA sequence was generated by siRNA Wizard V3.1 Online.

shRNA Oligo Insert / Sequence 5' – 3'
Sense mPinX1 shRNA oligo: CGCGTCCCC**AAGAAGAAAGTTTCCAGATAA**ttcaagaga **TTATCTGGAAACTTTCTTCTT**TTTTTGGAAAT
Antisense mPinX1 shRNA oligo: CGATTTCCAAAAA**AAGAAGAAAGTTTCCAGATAA**ttcaagaga **TTATCTGGAAACTTTCTTCTT**GGGGA
Sense Scrambled mPinX1 shRNA oligo: CGCGTCCCC**GAAACAGATAATAGACAGTTA**ttcaagaga **TAACTGTCTATTATCTGTTTC**TTTTTGGAAAT
Antisense Scrambled mPinX1 shRNA oligo: CGATTTCCAAAAA**GAAACAGATAATAGACAGTTA**ttcaagaga **TAACTGTCTATTATCTGTTTC**GGGGA

Table 2.5 List of shRNA Oligo Insert for pLVTHM subcloning. The oligos were designed according to Tronolab. Annealing the sense and antisense oligos would raise a 5' MluI sticky end and a 3' ClaI sticky end indicated by underline. The **bolded** sequences are showing the sense and antisense siRNA which would anneal to form a hairpin after transcription with the loop shown in small letters.

2.7 Lentiviral vector-mediated gene transfer to mESCs

2.7.1 Lentivirus packaging

$2X10^6$ HEK293FT at early passage were plated on T25 flask a day before transfection in 3ml medium. DNA constructs and the packaging plasmids (psPAX2 and pMD2.G) were co-transfected into the HEK293FT cells as described below.

Two reaction mixtures were prepared in 15ml falcon; both contained 0.63ml OPTI-MEM (Invitrogen). 4.2μg psPAX2, 1.05μg pMD2.G and 2.1μg DNA construct were incubated with OPTI-MEM in the first falcon; while 21μl lipofectamine 2000 was incubated with OPTI-MEM in the second falcon. The two reaction mixtures

were allowed to incubate at room temperature for 5 minutes. They were mixed gently together and stood at room temperature for another 20 minutes. At the same time, spent medium of HEK293FT was replaced with 3ml of OPTI-MEM medium. After 20 minutes, the reaction mixture was added to the HEK293FT cells and incubated at 37 ^{o}C for 6 hours. OPTI-MEM I medium with the complexes was changed back to normal medium after 6 hours and lentiviruses were collected at 24, 48 and 72 hrs post-transfection.

2.7.2 Checking of successful transduction by lentivirus in HEK cells

On day 1, 1x10^{4} HEK293FT cells were plated on each well of 24-well plate in 0.5ml medium. On the next day, each well of the cells were transduced with 200μl of virus and topped up with 200μl of medium with 6μg/ml polybrene. On day 4, the cells were seen under fluorescent microscope to investigate the transduction efficiency, and the batch of virus which gave the highest transduction efficiency and brightest green fluorescent signal were chosen for transduction in mESCs.

2.7.3 Multiple transductions to mESCs

mESCs at passage 8 were used for virus transduction. On day 0, 1x10^{4} mESCs were seeded on 12 well plates with MEFs. On day 1, 200μl of virus, 200μl of medium and

6μg/ml polybrene were added to each well. This transduction was repeated two more times at 12 hours and 24 hours after the first round of transduction. 12 hours after the final round of transduction, all viruses containing medium was removed and 500μl of fresh medium was added to each well for maintenance.

2.7.4 Selection of positive clones

Clones with brightest green fluorescent signal and morphology at undifferentiated state were selected. They were picked by glass pipettes in sterile hood under Lecia MZ95 microscope. The clones were washed with PBS and centrifuged at 3000rpm for 5 minutes. They were trypsinized for 3 minutes 37°C and gently pipetted up and down for dispersion into single cells. Fresh medium were then added and the cells were centrifuged for 5 minutes at 3000rpm. Supernatant was discarded, and the cells were resuspended in fresh medium and placed into 24-well plates seeded with MEFs.

2.7.5 Monoclonal establishment

After the single cells had formed observable clones, independent clones with bright green fluorescent signal were picked by glass pipettes in hood under Leica MZ95 microscope. Each clone were gently dispersed by pipetting up and down and seeded to a 24-well. The independent clones were expended and cultured as usual with some

frozen up as stocks. For Pwpi-PinX1 stable cell line, 4 clones were selected. For Pwpi-PinX1t, Pwpi only, shPinX1 and shScrambed stable cell lines, 3 clones were selected.

2.8 Total RNA preparation, Reverse Transcription (RT) and Quantitative Polymerase Chain Reaction (qPCR)

Total RNA was extracted using Trizol reagent (Invitrogen) according to manufacturer's protocol. Extracted RNA was dissolved in DNase/RNase-free water (Gibco) and quantified by measuring absorbance at 260nm and 280nm (Thermo Scientific, NanoDrop ND-1000). Integrity of extracted RNA was confirmed by the presence of 28s and 18s rRNA by electrophoresis in 1% agarose gel. The resulting RNA was DNase I (Invitrogen)-treated and subjected to RT using SuperScript III reverse transcriptase (Invitrogen). RT was confirmed successful by PCR using the RT products as the templates and the GAPDH gene primers. For real-time qPCR, Power SYBR GREEN PCR Master Mix (Applied Biosystems) was used and qPCR reactions were performed with 7500 Fast Real-time PCR system (Applied Biosystems). Each reaction was performed at least in triplicate under the following conditions: 50°C for 2 minutes; 95°C for 10 minutes; 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Melting curve analysis and electrophoresis of PCR

products were performed to verify the authenticity of PCR products. For checking amplification efficiency by individual primer pairs, standard curve experiments were performed. The starting quantity of mESCs cDNA was 500ng and serial factor was 1:10. Five points were used in the standard curve. Primer pairs with PCR efficiency of 90–110 % were used for further experiments.

Fold-changes in the relative mRNA expression of the target gene were determined using the $2^{-\Delta\Delta Ct}$ method in which the target gene was normalized to the housekeeping gene β-actin and the relative expression levels of different genes in experimental groups were normalized to that in control group.

Primer	**Sequences**	**Product Size (bp)**	**GenBank accession no.**
mPinX1			
5' Primer	CCACGGTCAGGAAACAGCAG	197	NM_028228.3
3' Primer	AGCCATCCTGAGCAAGCTTC		
mPinX1t			
5' Primer	AAAGGGAAGGATCTGTCCTC	197	/
3' Primer	CCATCTCCAGACAATCTTAATC		

Table 2.6 List of primers used in RT-qPCR. The sequence of primer, product size and the GenBank accession number of the gene were shown in the table.

2.9 Immunocytochemistry

Cells were trypsinized and seeded onto glass slides pre-coated with gelatin. After the

cells were seated onto the glass slides, which took around 4-5 hours, they were washed with PBS and fixed with freshly prepared 4% PFA in PBS at room temperature for 30 minutes. After that the cells were washed twice with PBS each for 15 minutes at room temperature, and then blocked with blocking buffer (0.5% (w/v) Non-fat dry milk (Bio-rad), 5% (v/v) Normal goat serum in PBS) at room temperature for 1 hour with gently shaking. The cells were then incubated with primary antibody diluted in blocking buffer overnight at 4 ^{o}C with gently shaking. On the next day, the cells were washed with washing buffer (0.1% Tween-20 (USB) in PBS) for 15 minutes. After 4 times of washing, the cells were stained with secondary antibody diluted in 1% normal goat serum in PBS for 1 hour at room temperature. After staining, the cells were washed with washing buffer for 15 minutes, and after 4 times of washing, the cells were counterstained with 40μg/ml propidium iodide with 100μg/ml RNase A for 15 minutes at room temperature if desired. The stained cells were observed and imaged with Leica SP5 Confocal Microscope and software.

Anti-body used in ICC	**Dilution**
Anti-Fibrillarin (Cell Signaling)	1:1000
Dylight 594 Goat Anti-Rabbit (Jackson)	1:1000

Table 2.7 List of anti-body and the specific dilution used in ICC experiments.

2.10 Western Blot

2.10.1 Total Protein Extraction

Cells were harvested, washed with PBS once and lysed in ice-cold RIPA buffer containing PBS with 1% NP-40, 0.5% sodium deoxycholate, 1% SDS, 1% protease inhibitor cocktail [Leupeptin (1μg/mL), Aprotinin (5μg/mL), PMSF (100μg/mL), sodium orthovanadate (100μg/mL), EGTA (200μg/mL) and EDTA (200μg/mL) and phosphatase inhibitor cocktail [NaF (250mM), β-glycerolphosphate (500μg/mL) and HEPES (50mM, pH 7.3)]. The cell lysate was incubated on ice for 10 minutes, and centrifuged at 16000g for 20 minutes at 4°C. The supernatant was collected and the protein concentration was estimated by Bradford assay (Bio-Rad Laboratories). Aliquots of protein were stored at -80°C until performing electrophoresis.

2.10.2 Measurement of Protein Concentration

To determine the protein concentration, 200μl Bradford reagent was mixed with 1μl of 10 fold diluted sample protein in a flat bottom 96-well plate in duplicate. Bovine serum albumin (BSA) standards of various concentrations were also included. After incubation for 5 minutes at room temperature, the absorbance reading of each well was taken by a microplate reader (Model 3550, Bio-Rad) at wavelength 595nm. Protein concentration of each sample was then determined directly from the BSA

standard curve.

2.10.3 SDS-PAGE and chemiluminescent detection

Aliquots of total protein were mixed in 1:1 ratio with 1x sample loading buffer containing 125mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 0.06% bromophenol blue and 10% β-mercaptoethanol. Depending on the molecular weight of target protein, samples were separated in 6–12.5% SDS polyacrylamide gel. Pre-stained Novex Sharp Standard marker (Invitrogen) was loaded in parallel as a standard molecular weight marker. The 4% stacking gel was electrophoresed at 70 volts for 30 minutes while the separating gel was increased to 100-120 volts for 1.5–2 hours. After electrophoresis, the gel was electrophoretically transferred onto PVDF membranes (Millipore) with a Trans-blot SD wet transfer tetra-cell (Bio-Rad) at 100 volts for 1.5-2 hours, duration of which depended on the size of target protein. Membranes were blocked for 1 hour at room temperature with 5% dried non-fat milk in Tris-buffered saline containing 0.1% Tween-20 (TBST). Incubation with primary antibodies was carried out at 4°C overnight. For the determination of PinX1 antibody specificity, antigenic peptides were pre-incubated with corresponding antibodies (10:1 w/w) in 500μl of PBS at room temperature for 2 hours before adding to 5% milk. After primary antibody probing, membranes were subjected to three washes in

TBST, each for 15 minutes with gentle shaking. Membranes were next incubated with HRP-conjugated secondary antibodies for 45 minutes at room temperature, followed by three 15-minute washes with TBST. Protein expression was detected by enhanced chemiluminescent substrate (ECL: Pierce) and protein bands were visualized by film exposure. TATA-box binding protein (TBP) or H3 were used as internal controls. The density of the bands was quantified using AlphaEaseFC software (Alpha Innotech).

Primary Anti-body used in Western blot	**Dilution**
Anti-Sox2 (Abcam)	1:1000
Anti-Oct4 (Santa Cruz)	1:400
Anti-Nanog (Abcam)	1:1000
Anti-Klf-4 (Abcam)	1:1000
Anti-PinX1 (Kindly provided by Prof. P.C. Shaw, CUHK)	1:1000
Anti-H3 (Abcam)	1:10,000
Anti-TBP (Abcam)	1:1000
Anti-β tubulin (Santa Cruz)	1:1000
Anti-Myc (Cell signalling)	1:1000

Table 2.8 List of primary anti-body and the specific dilution used in western blot.

Secondary Anti-body used in Western blot	**Dilution**
Goat Anti-Rabbit IgG, HRP conjugated (Dako)	1:5000
Rabbit Anti-Mouse IgG, HRP conjugated (Dako)	1:5000
Goat Anti-Rabbit IgG, HRP conjugated (Invitrogen)	1:10,000

Table 2.9 List of secondary anti-body and the specific dilution used in western blot.

2.11 Co-immunoprecipitation

On day 1, 2×10^5 HEK cells were plated on each well of 6-well plate. For each well, 1μg of mTERT recombinant plasmid was mixed with 1μg of mPinX1 or mPinX1t recombinant plasmid, and 6μl of Lipofectamine 2000 was used. Transfection was done according to section 2.5. 48hrs after transfection, medium was removed and the cells were washed with 1ml PBS. The cells were harvested by scarper with 500μl IP buffer (50mM Tris pH 7.6, 150mM NaCl, 1mM EDTA, 1% Triton X100) in the presence of protease inhibitors and phosphatase inhibitors (1μg/ml Leupetin, 5μg/ml Aprotonin, 100μg/ml PMSF, 1mM Sodium Orthovanadate, 1mM EGTA, 1mM EDTA, 1mM NaF, 2mg/ml β-glycerolphosphate). The cells were sonicated for a few seconds followed by centrifugation at 13,000rpm at 4°C for 10 minutes. 200μl of supernatant was topped up to 500μl with IP buffer and anti-body at a specific dilution. The mixture was incubated at 4°C overnight with gently shaking.

On the next day, 15μl of protein A-agarose beads (Sigma) was added to each reaction tube and incubated at 4°C for 1.5 hours with gently shaking. After that, the beads were spun down at 13,000rpm for 10 seconds, supernatant was removed and the beads were washed with IP buffer. After 3 times of washing, 30μl loading dye was added to each tube and the beads were boiled for 5 minutes followed by SDS-PAGE and chemiluminescent detection according to section 2.10.3.

Anti-body used in pull down	Dilution
Anti-Myc (Cell Signaling)	1:500
Anti-HA (Sigma)	1:500

Table 2.10 List of anti-body and the specific dilution used in pull down during Co-IP.

2.12 Telomere Repeat Amplification Protocol (TRAP) Assay

The TRAP assay was done with Telo*TAGGG* telomerase PCR ELISA (Roche) according to the manufacturer's instructions as follows. Briefly, the cells were harvested and counted using a hemocytometer. 2×10^5 cells were transferred to a new eppendorf and centrifuged at 3000g for 10 minutes at 4 °C. Supernatant was removed and the cell pellet was resuspended with 200µl lysis reagent and incubated on ice for 30 minutes. After that, the lysate was centrifuged at 16,000g for 20 minutes at 4 °C. 3µl of the supernatant (cell extract) was mixed with 25µl of reaction mixture and topped to 50µl with autoclaved double distill water. The tubes were then transferred to a thermo cycler and performed a combined primer elongation/ amplification reaction by the following protocol: Primer elongation at 25 °C for 30 minutes, then telomerase inactivation at 94 °C for 5 minutes, followed by 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 50 °C for 30 seconds and polymerization at 72 °C for 90 seconds, and at last a final extension at 72 °C for 10 minutes.

After elongation and amplification, the PCR product was hybridized to a digoxigenin

(DIG)-labeled, telomeric repeat-specific detection probe and immobilized to a streptavidin-coated microplate. Firstly, 20µl of the denaturation reagent was transferred to each new reaction tube, and then 5µl of the PCR product was added and incubated at room temperature for 10 minutes. Afterwards 225µl of hybridization buffer was added per tube and mixed thoroughly by vortexing. 100µl of the mixture was transferred per well of the precoated MP modules supplied, the wells were then covered with the self-adhesive cover foil and incubated at 37°C for 2 hours with shaking at 300rpm. After incubation, the solution was removed completely and the wells were washed 3 times with 250µl washing buffer. After removing all washing buffer, 100µl of Anti-DIG-POD working solution was added per well and incubated at room temperature for 30 minutes in dark with shaking at 300rpm. Again all the solution was removed completely and the wells were washed with 250µl of washing buffer for 5 times. Then 100µl of TMB substrate solution was added per well and incubated at room temperature for 20 minutes with shaking at 300rpm. Without removing the reacted substrate, 100µl stop reagent was added to stop color development. By using a microplate reader, the absorbance of the samples at 450nm and 690nm (as a reference wavelength) was measured.

2.13 Cell cycle analysis

The collected cells were washed with PBS and centrifuged at 5000 rpm at 4°C for 5 minutes and fixed with 70% ethanol at 4°C for 30 minutes. The cells were centrifuged at 5000 rpm at 4°C for 10 minutes, washed with PBS and centrifuged again at 5000 rpm at 4°C for 10 minutes to remove the fixative. Then the cells were re-suspended in 1ml freshly prepared PI DNA staining buffer (4μg/ml PI, 10μg/ml Rnase A in PBS) and incubated in dark at 37°C for 30 minutes. The stained cells were then analyzed by a flow cytometer (FACSCanto, BD Biosciences) with the FACSDiva software (BD Biosciences) to measure the DNA content. The cell cycle distribution patterns of the cells in G0/G1, S and G2/M phases were determined by the MODFIT software (Verity Software House).

2.14 MTT assay

96-well plates were coated with gelatin. After that each clone were seeded to 6 wells at a density of 1×10^4 cells per well. 24 hours after the medium was discarded and 100μl of medium with 1mg/ml MTT were added to each well, followed by 4 hours of incubation at 37°C. After the incubation, 100μl of DMSO was added to each well to dissolve the formazan crystal. OD_{570} was then measured with OD_{690} as reference in Tecan Infinite 2000.

2.15 Statistical analysis

The results were expressed as mean ± SEM. Statistical significance between two groups of means was determined using unpaired Student's t-test. $P < 0.05$ was considered to be statistically significant.

CHAPTER 3 RESULTS

3.1 mPinX1t was discovered in mESCs

In a PCR experiment, a pair of primers flanking the coding sequence of mPinX1 was used to amplify the mPinX1 gene in undifferentiated mESCs and their differentiation derivatives. An additional band (~1100 bp) on top of the expected mPinX1 band (999 bp) was found (Fig.3.1). The sequence of the additional band was sequenced and 111 additional nucleotides, when compared with the mPinX1 coding sequence, was found. This additional 111bp was blast-searched and was found to be located in Locus NT_039606.7, where the mPinX1 gene is located. This 111bp was found in between the two exons of the mPinX1 gene. This newly discovered transcript variant was predicted to code for a novel protein of 170 amino acids because a stop codon was found in the additional nucleotides. It is named mPinX1t where 't' stands for the 'truncated' form of mPinX1.

Both sequences were then subcloned into pTriEx4-neo plasmid so that the proteins, when translated, would be tagged with His-tag at the N-terminal (Fig.3.2). By transfecting the two plasmids into HEK cells and using western blot to probe for the His-tagged protein, the size of both mPinX1 and mPinX1t could be deduced and compared. It was found that the size of His-mPinX1 is around 55kDa, while the size

of His-mPinX1t is around 30-35kDa. (Fig.3.3) The size of His tag sequence was around 8kDa, by subtracting the mass of His tag, the deduced mPinX1 and mPinX1t sizes were around 47kDa and 22-27kDa respectively.

Figure 3.1

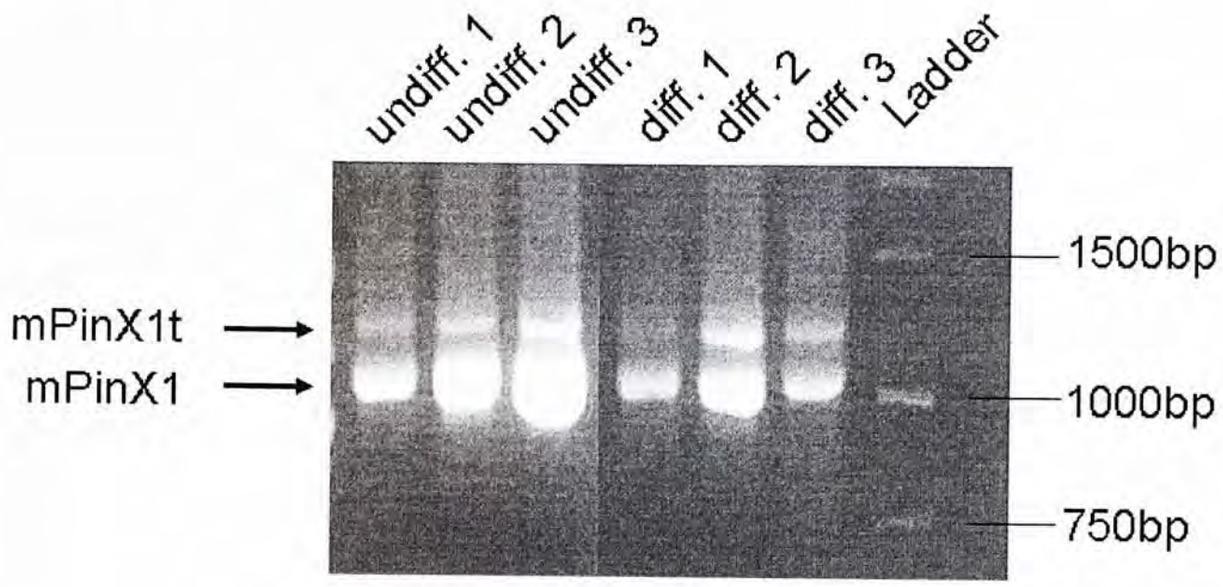


Figure 3.1 Gel photo showing the expression of mPinX1 and mPinX1t in undifferentiated mESCs (undiff. 1-3) and their Day 7+25 differentiation derivatives (diff. 1-3). By using a pair of primer flanking the coding sequence of mPinX1, both mPinX1 and mPinX1t could be detected in the two types of cells.

Figure 3.2

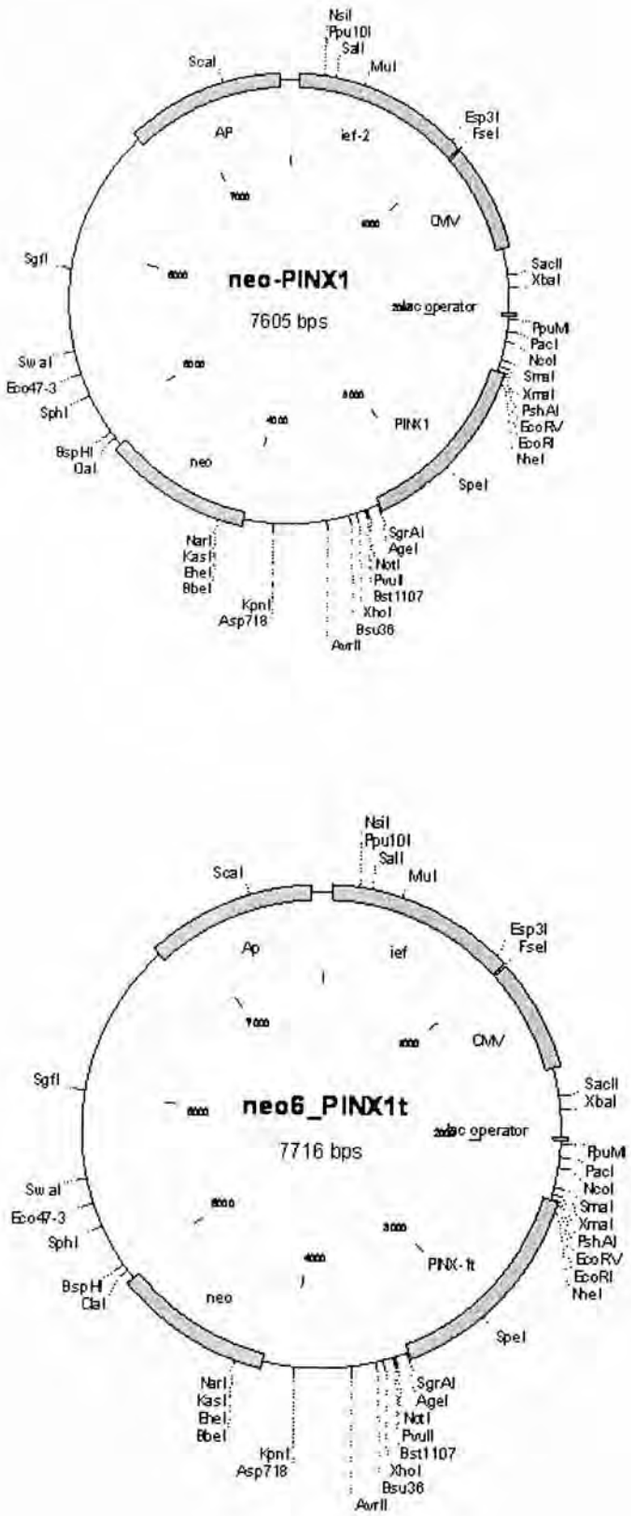


Figure 3.2 The vector maps of (upper panel) pTriEx4-neo-mPinX1 and (lower panel) pTriEx4-neo-mPinX1t.

Figure 3.3

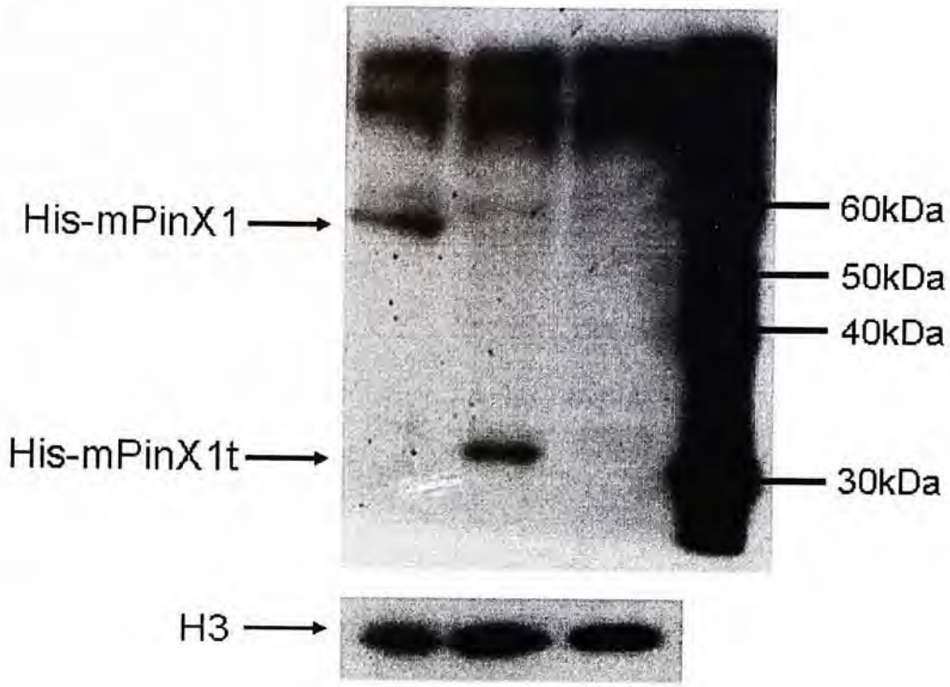


Figure 3.3 Western blot showing the expression of His-mPinX1 and His-mPinX1t when pTriEx4-neo-mPinX1 and pTriEx4-neo-mPinX1t plasmids were transfected into HEK cells. (From left) Lane 1: HEK transfected with pTriEx4-neo-mPinX1. Lane 2: HEK transfected with pTriEx4-neo-mPinX1t. Lane 3: HEK transfected with pTriEx4-neo vector only. Lane 4: Ladder.

3.2 mPinX1 and mPinX1t were expressed at transcriptional level in the inspected mouse tissues

After knowing that mPinX1 and mPinX1t are expressed in mESCs and their derivatives (Fig.3.4 & Fig.3.5), we would like to know if they are expressed in normal mouse tissues. RT-qPCR was done on mPinX1 and mPinX1t with 12 mouse tissues, undifferentiated mESCs and their differentiation derivatives. Both mPinX1 and mPinX1t could be detected in the 12 mouse tissues investigated at transcriptional level. All the gene expression levels were normalized with that of Day 7+25 differentiation derivatives. mPinX1 expression was found to be higher in liver, while mPinX1t expressed at a higher level in testis and uterus. (n=1-2, Fig.3.4 & Fig.3.5)

Figure 3.4

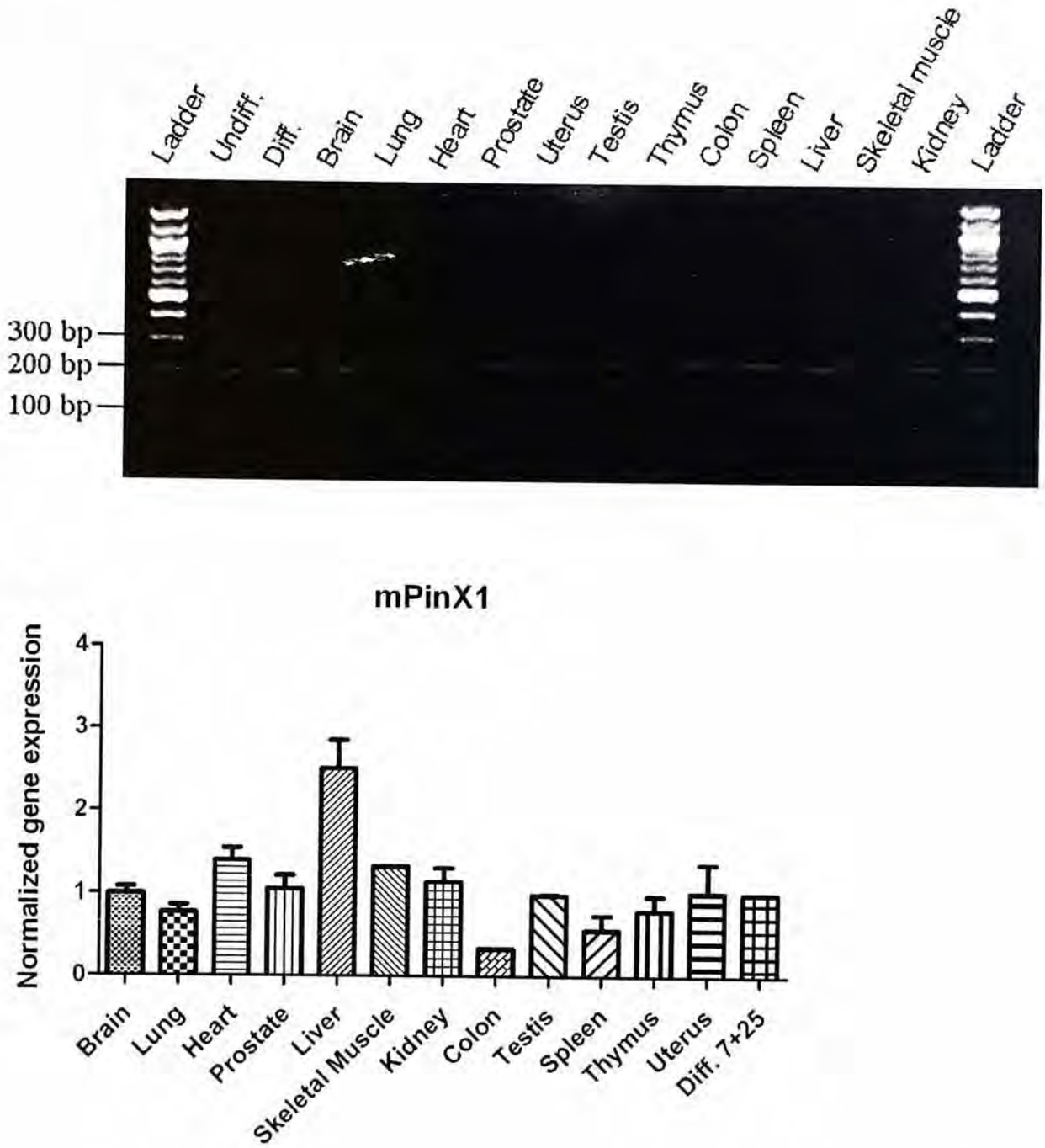


Figure 3.4 (Upper panel) RT-PCR result showing the expression of mPinX1 in 12 mouse tissues, undifferentiated mESCs and their Day 7+25 differentiation derivatives. mPinX1 transcript could be detected in all the mouse tissues and cell types investigated. (Lower panel) Bar chart showing the RT-qPCR result of mPinX1 transcript expression in 12 mouse tissues, normalized with Day 7+25 differentiation derivatives group. β-actin was used as the loading control. mPinX1 expression was found to be highest in liver. Values are mean ± S.E.M. of 1-2 experiments.

Figure 3.5

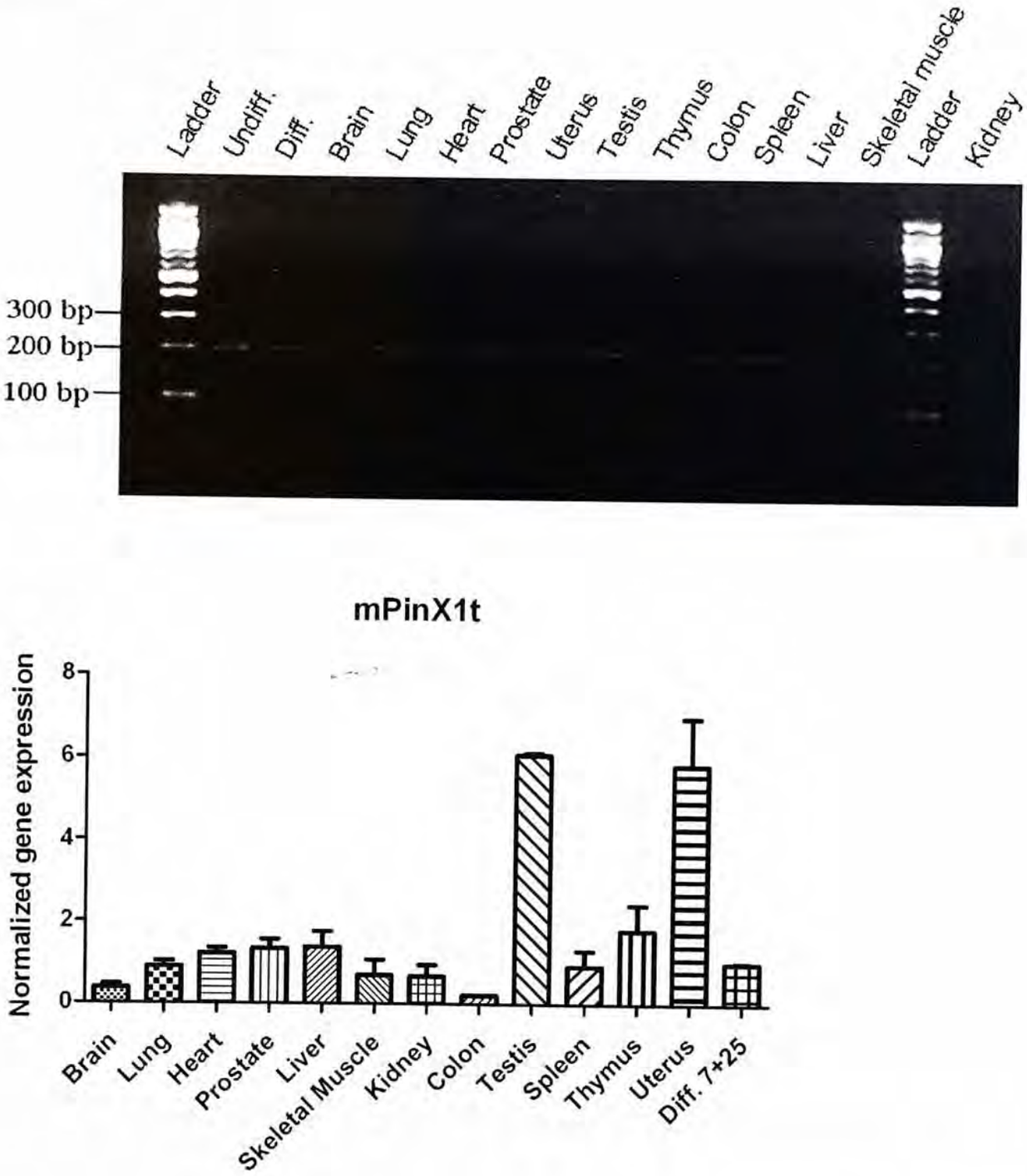


Figure 3.5 (Upper panel) RT-PCR result showing the expression of mPinX1t in 12 mouse tissues, undifferentiated mESCs and their Day 7+25 differentiation derivatives. mPinX1t transcript could be detected in all the mouse tissues and cell types investigated. (Lower panel) Bar chart showing the RT-qPCR result of mPinX1t transcript expression in 12 mouse tissues, normalized with Day 7+25 differentiation derivatives group. β-actin was used as the loading control. mPinX1t expression was found to be highest in testis and uterus. Values are mean ± S.E.M. of 1-2 experiments.

3.3 Expression of mPinX1 and mPinX1t changed upon differentiation

Previous studies showed that telomerase activity will decrease dramatically upon differentiation [2, 44, 78, 80], it is possible that the expressions of its regulators also change as development proceeds. To see if the expression of the two genes will change upon differentiation, RT-qPCR was done on undifferentiated mESCs and their differentiation derivatives (Day 7+25). It was found that the mRNA expression of mPinX1 decreased (1.00 ± 0.17 Vs 0.48 ± 0.06, $n=7$, $p<0.05$) while that of mPinX1t increased (1.00 ± 0.13 Vs 1.46 ± 0.16, $n=7$, $p<0.05$) upon differentiation. (Fig.3.6) Western blot using an in-house antibody against PinX1 revealed multiple bands in both the undifferentiated and differentiation derivatives; peptide-preincubation experiment showed that bands at around 45 and 70 kDa are the specific bands (Fig. 3.8). The size of mPinX1 matched with the predicted size, which is around 47kDa in both undifferentiated mESCs and its differentiation derivatives. The protein expression of mPinX1 decreased upon differentiation (2.84 ± 0.06 in mESCs Vs 1.00 ± 0.08 in differentiation derivatives, $n=7$, $p<0.01$, Fig.3.8). This suggests that both of them might play a role in the mechanisms involved in/ induced by differentiation.

Apart from the findings that the expressions of both genes change during differentiation, to see if the endogenous levels of the two genes are different at

particular stages, the transcript expression levels of mPinX1 and mPinX1t were also compared in the undifferentiated and differentiated stages. mPinX1 level was much higher than that of mPinX1t in both the undifferentiated stage (1.00 ± 0.00 for mPinX1 Vs 0.05 ± 0.01 for mPinX1t, $n=7$, $p<0.001$) and the differentiated stage (Day 7+25) (1.00 ± 0.00 for mPinX1 Vs 0.12 ± 0.02 for mPinX1t, $n=7$, $p<0.001$) (Fig.3.7).

Figure 3.6

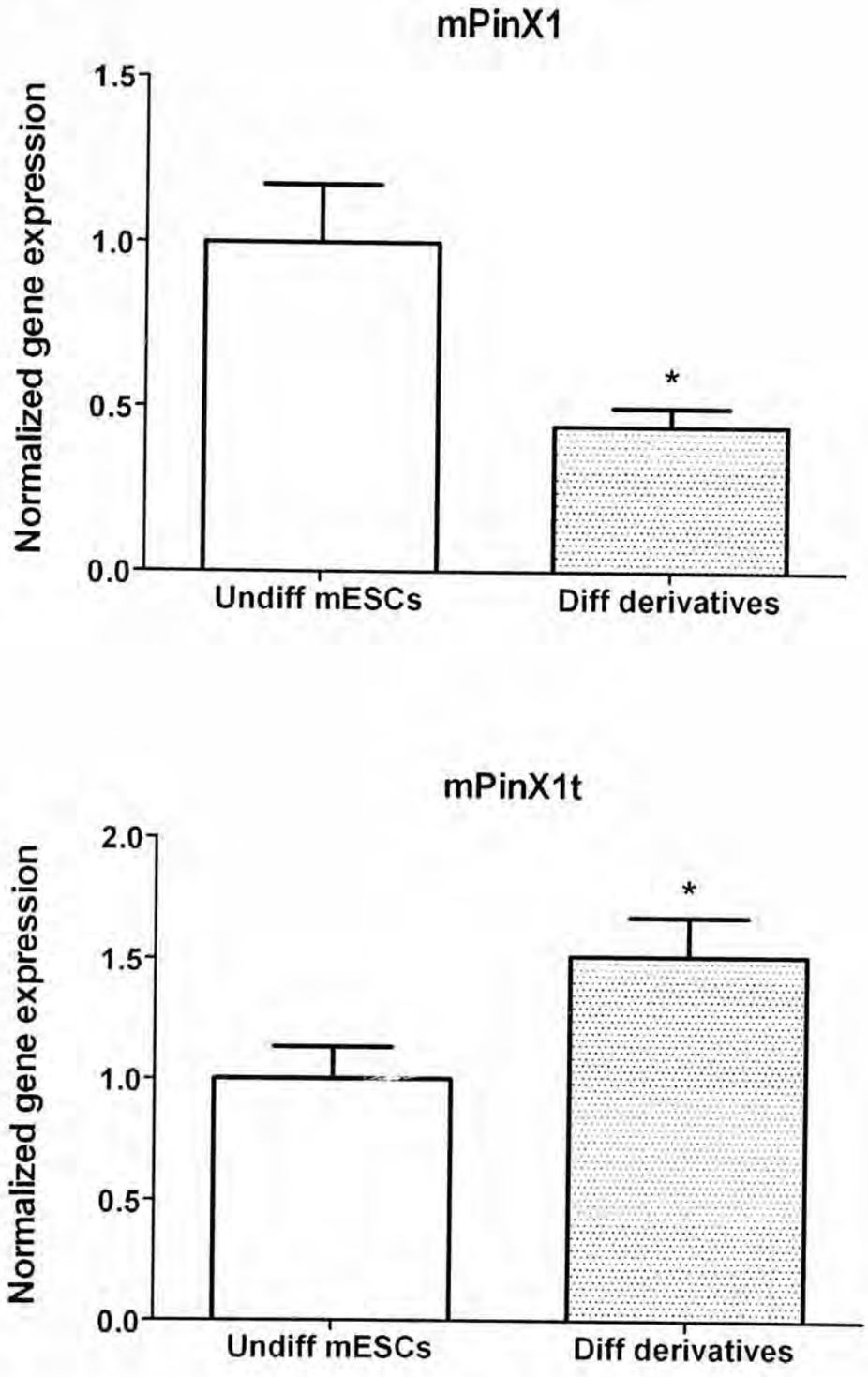


Figure 3.6 RT-qPCR results showing the gene expressions of (upper panel) mPinX1 and (lower panel) mPinX1t in undifferentiated mESCs and their Day 7+25 differentiation derivatives. The gene expressions were normalized with undifferentiated mESCs group. β-actin was used as the loading control. mPinX1 expression decreased upon differentiation, while mPinX1t expression increased upon differentiation. Values are mean ± S.E.M. of 7 experiments. *$p<0.05$ Vs undifferentiated mESCs group.

Figure 3.7

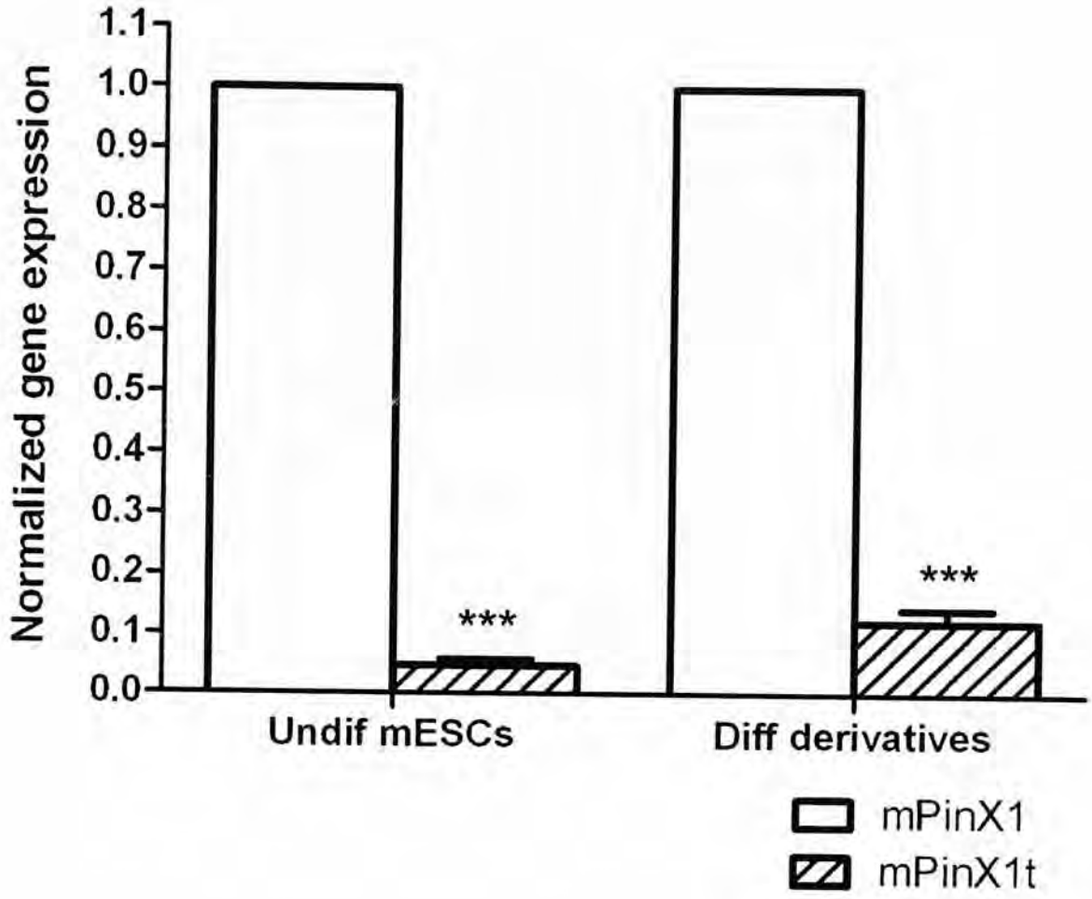


Figure 3.7 RT-qPCR result comparing the gene expression of mPinX1 and mPinX1t in undifferentiated mESCs and their Day 7+25 differentiation derivatives. The gene expression level of mPinX1t was normalized with that of mPinX1. β-actin was used as the loading control. mPinX1 expression was much higher than that of mPinX1t in both groups of cells. Values are mean ± S.E.M. of 7 experiments. ***$p<0.001$ Vs mPinX1.

Figure 3.8

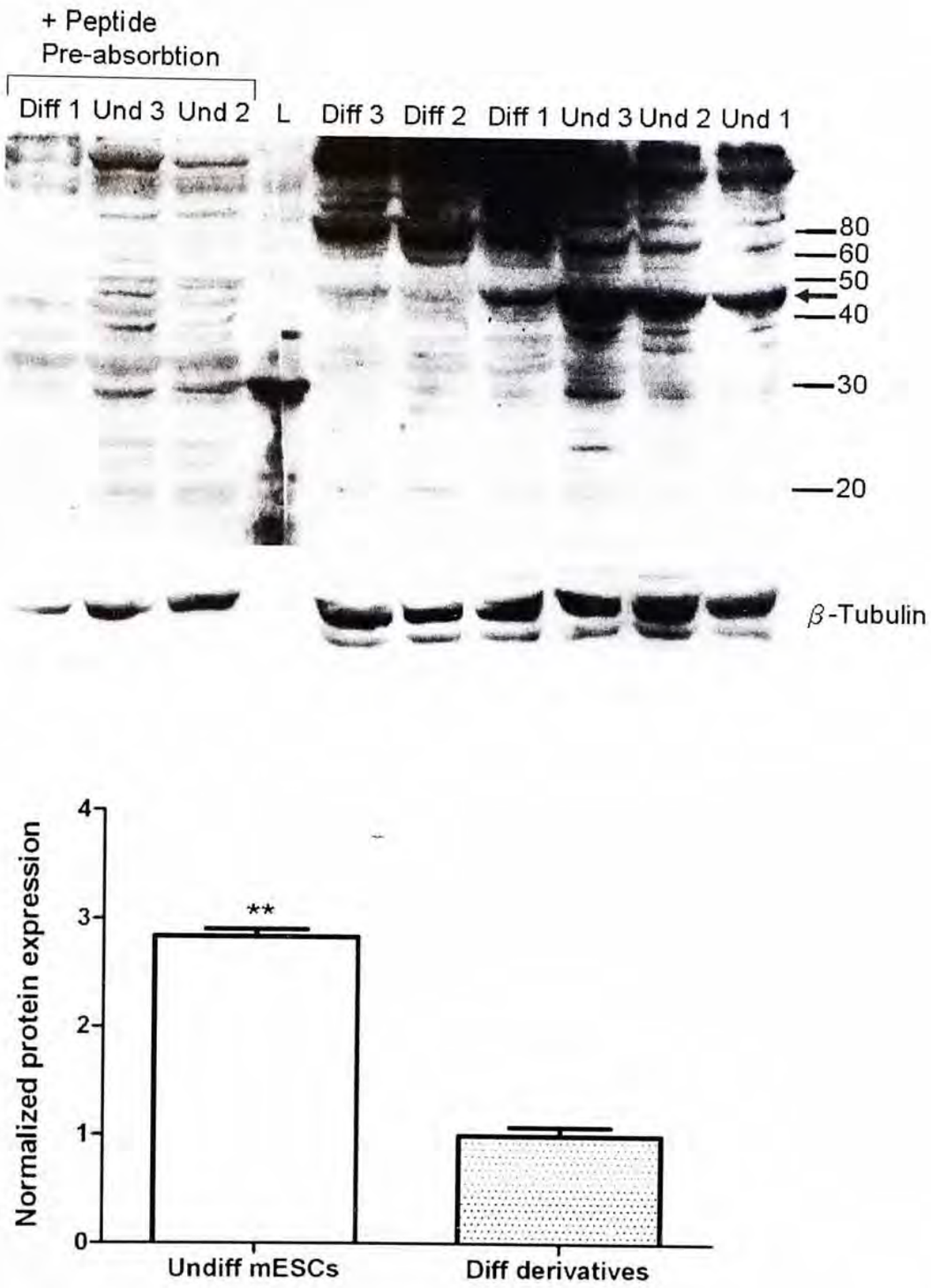


Figure 3.8 (Upper panel) Western blot showing the protein expression of mPinX1 in undifferentiated mESCs and their Day 7+25 differentiation derivatives. β-tubulin was used as the loading control. Pre-absorption with peptide was done to make sure the bands were specific. (Lower panel) Bar chart showing the mPinX1 expression normalized with that of Day 7+25 differentiation derivative groups. The mPinX1 level was higher in undifferentiated mESCs than in the Day 7+25 differentiation derivatives. Values are mean ± S.E.M. of 3 experiments. **$p<0.01$ Vs Day 7+25 diff.

3.4 mPinX1 and mPinX1t were both located in the nucleolus and the nucleoplasm in undifferentiated mESCs

Previous studies showed that the hPinX1 mainly resides in the nucleolus [53, 54, 68]; however, the subcellular localization of the mouse homolog is unknown. To investigate the subcellular localization of mPinX1 and mPinX1t, sub-cloning of the two genes into the pWPXL plasmid was performed. Transcription and translation of the plasmids will result in GFP fusion proteins (Fig.3.9). The constructs were then transfected into mESCs and examined under confocal microscope with staining of nucleus with PI and nucleolus with fibrillarin. mPinX1 was found to mainly localize in the nucleolus, with some localizing in the nucleoplasm, similar to what has been observed for hPinX1 [54] (Fig.3.10). Similarly, mPinX1t also mainly resided in the nucleolus, with some resided in the nucleoplasm (Fig.3.11).

Figure 3.9

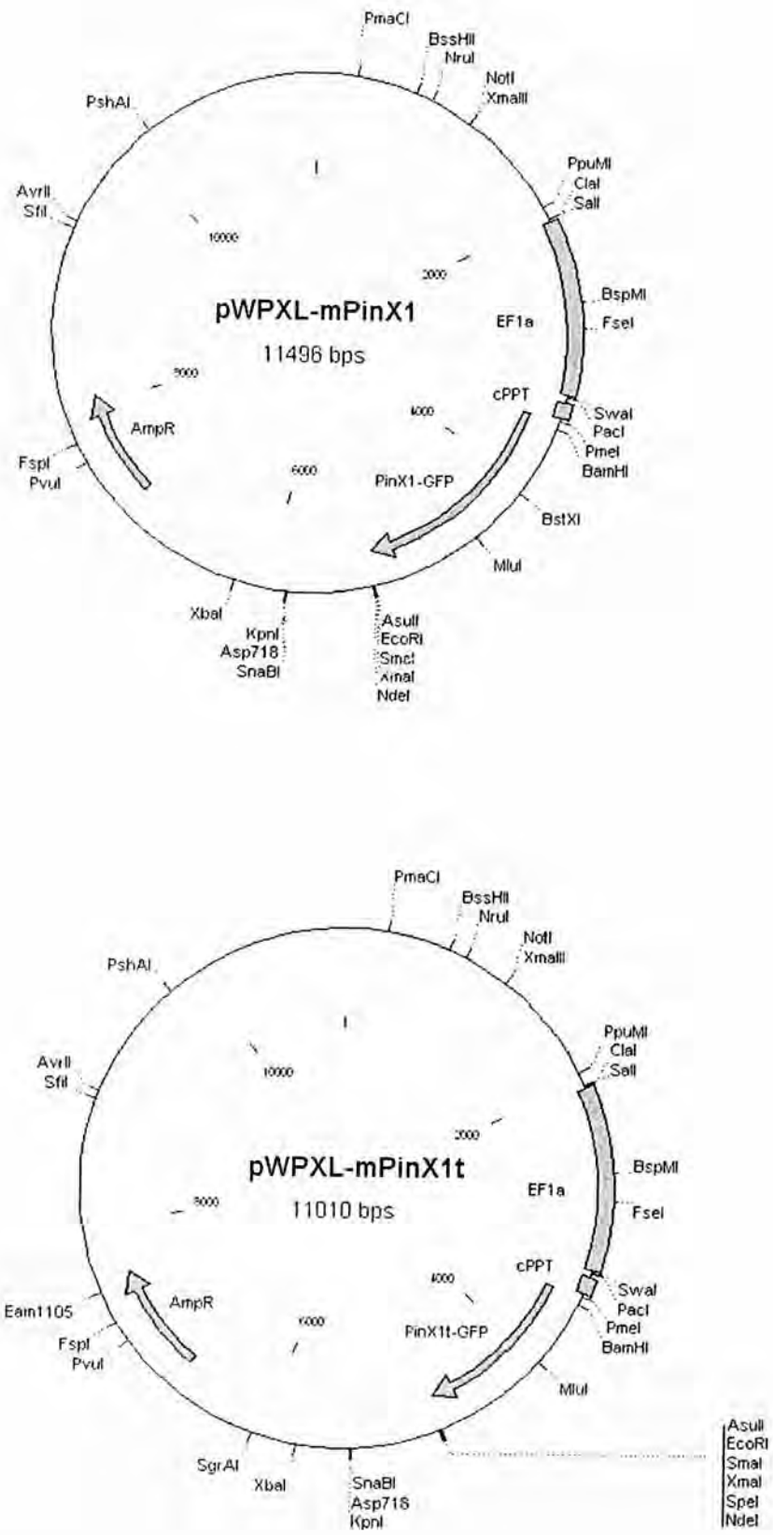


Figure 3.9 The vector maps of (upper panel) pWPXL-mPinX1-GFP and (lower panel) pWPXL-mPinX1t-GFP.

Figure 3.10

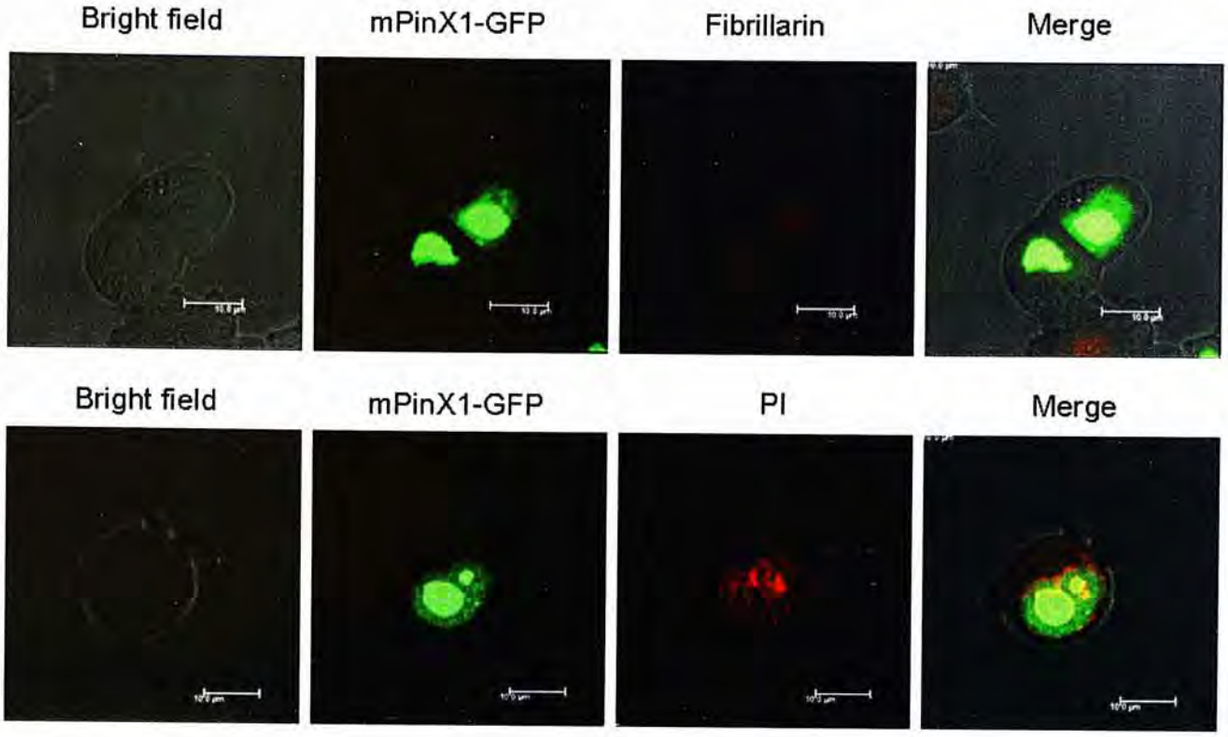


Figure 3.10 Sub-cellular localization of mPinX1 in mESCs by confocal microscopy. (From left to right) The 4 diagrams in each figure represent the cells under bright field, green signal given by GFP tagged mPinX1, red signal given by fibrillarin which stained the nucleolus or red signal given by PI which stained the nucleus, and the merged red and green signal. Overlapping of the green and both red signals indicates mPinX1-GFP resided in both nucleoplasm and nucleolus.

Figure 3.11

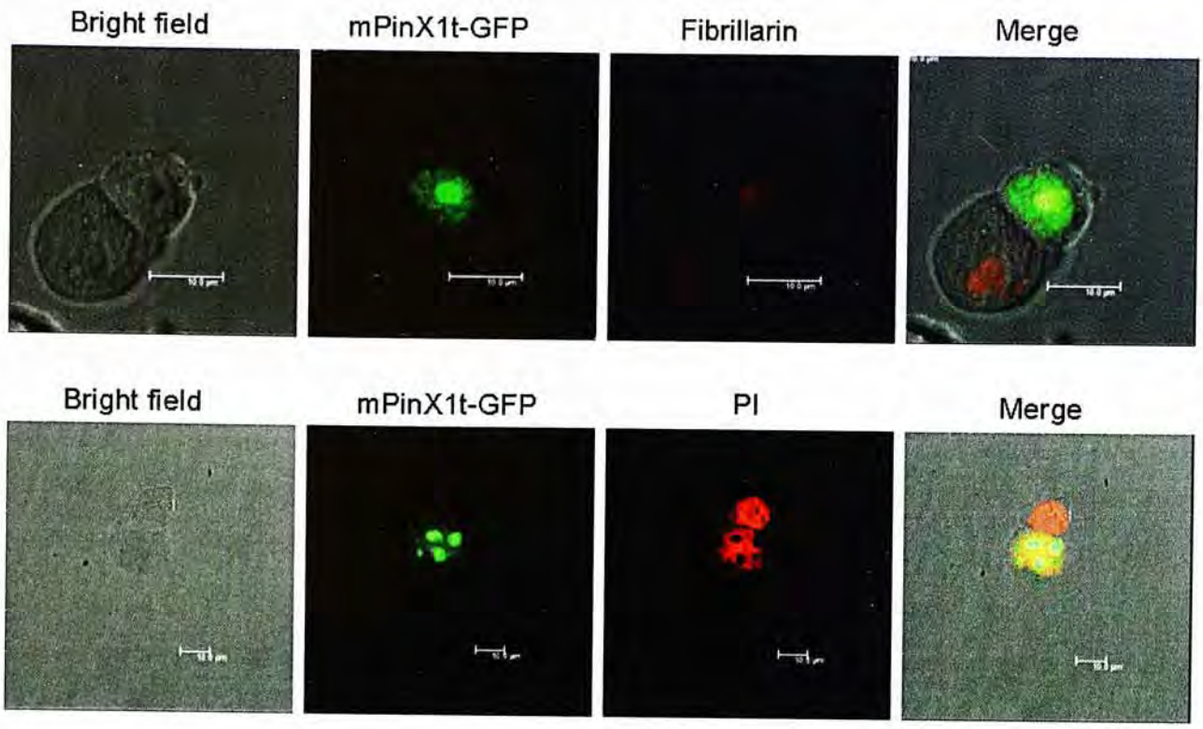
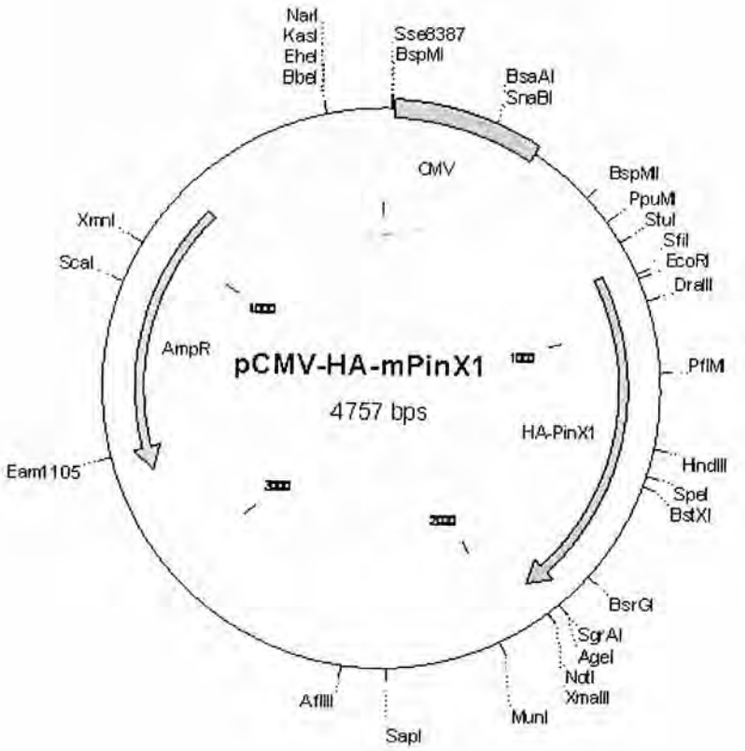


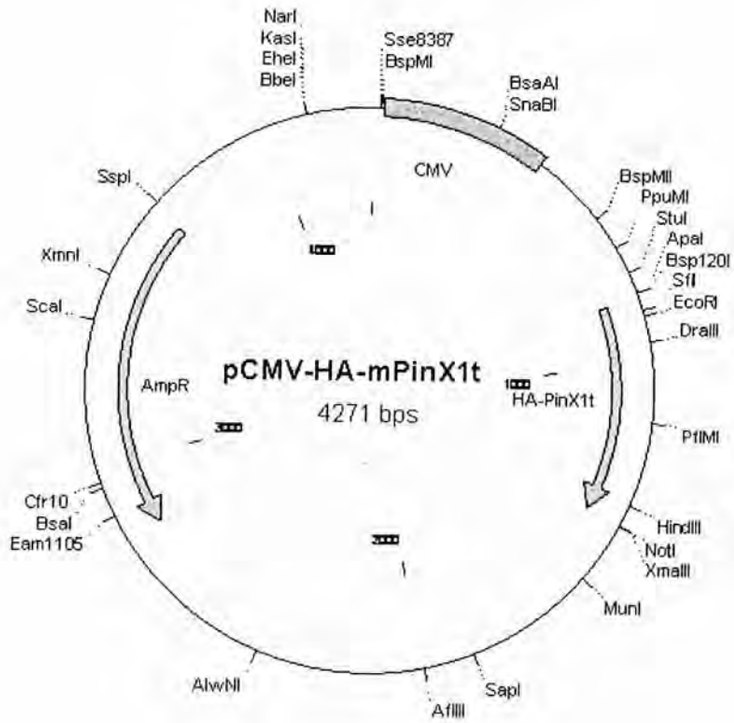
Figure 3.11 Sub-cellular localization of mPinX1t in mESCs by confocal microscopy. (From left to right) The 4 diagrams in each figure represent the cells under bright field, green signal given by GFP tagged mPinX1t, red signal given by fibrillarin which stained the nucleolus or red signal given by PI which stained the nucleus, and the merged red and green signal. Overlapping of the green and both red signals indicates mPinX1t-GFP resided in both nucleoplasm and nucleolus.

3.5 Co-immunoprecipitation (Co-IP) of mPinX1 and mPinX1t with mTERT

Previous studies showed that hPinX1 directly bind with hTERT [112]. To investigate if mPinX1 and/or mPinX1t regulate telomerase activity using similar mechanism in mESCs, Co-IP of mPinX1 and mTERT (or mPinX1t and mTERT) was carried out to see if mPinX1 and/or mPinX1t interact with mTERT. pCMV-HA-mPinX1, pCMV-HA-mPinX1t and pCMV-Myc-mTERT were made (Fig.3.12). pCMV-HA-mPinX1 + pCMV-Myc-mTERT, or, in another set of experiment, pCMV-HA-mPinX1t + pCMV-Myc-mTERT, were co-transfected into HEK cells. The cells were collected 48 hours after transfection and subjected to Co-IP. In both experiments, Myc-mTERT was pulled down by anti-Myc antibody. HA-mPinX1 and HA-mPinX1t in the pull-down lysate were detected by anti-PinX1 antibody. In the control experiments, Myc-mTERT was pulled down by anti-Myc antibody (Fig.3.13 & Fig.3.14). Both HA-mPinX1 and HA-mPinX1t could be found in the pull-down lysates and detected by the anti-PinX1 antibody (Fig.3.13 & Fig.3.14). In the negative control, empty myc-vector was co-transfected with either pCMV-HA-mPinX1 or pCMV-HA-mPinX1t. Using anti-Myc to pull down did not result in the detection of mPinX1 or mPinX1t in the pull-down lysate. Altogether, the results suggest that both mPinX1 and mPinX1t could interact with mTERT. (n=3-5, Fig.3.13 & Fig.3.14).

Figure 3.12


NarI
KasI
EheI
BbeI
Sse8387
BspMI
BsaAI
SnaBI
CMV
BspMII
PpuMI
StuI
SfiI
EcoRI
DraIII
PflMI
HindIII
SpeI
BstXI
BsrGI
SgrAI
AgeI
NotI
XmaIII
MunI
SapI
AflIII
Eam1105
ScaI
XmnI
AmpR
pCMV-HA-mPinX1
4757 bps
HA-PinX1


NarI
KasI
EheI
BbeI
Sse8387
BspMI
BsaAI
SnaBI
CMV
BspMII
PpuMI
StuI
ApaI
Bsp120I
SfiI
EcoRI
DraIII
PflMI
HindIII
NotI
XmaIII
MunI
SapI
AflIII
AlwNI
Cfr10
BsaI
Eam1105
ScaI
XmnI
SspI
AmpR
pCMV-HA-mPinX1t
4271 bps
HA-PinX1t

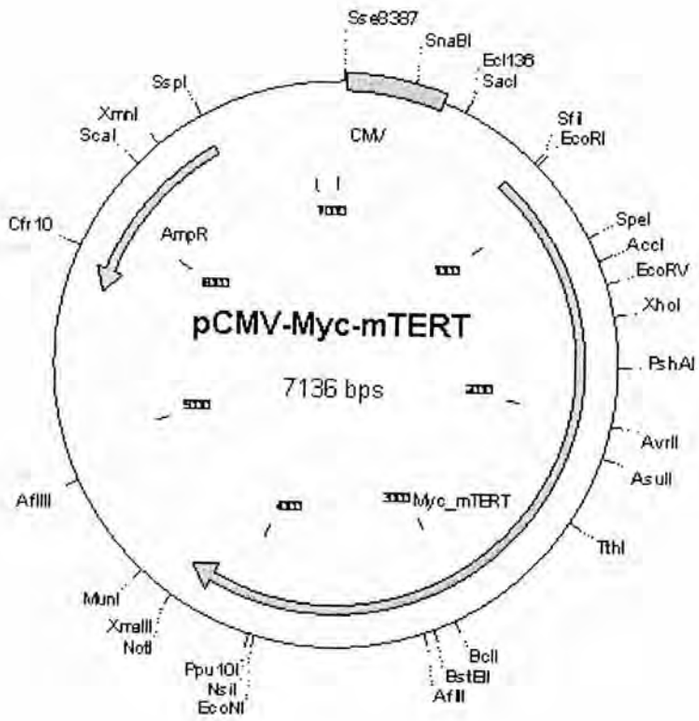


Figure 3.12 The vector maps of (upper panel) pCMV-HA-mPinX1, (middle panel) pCMV-HA-mPinX1t and (lower panel) pCMV-Myc-mTERT.

Figure 3.13

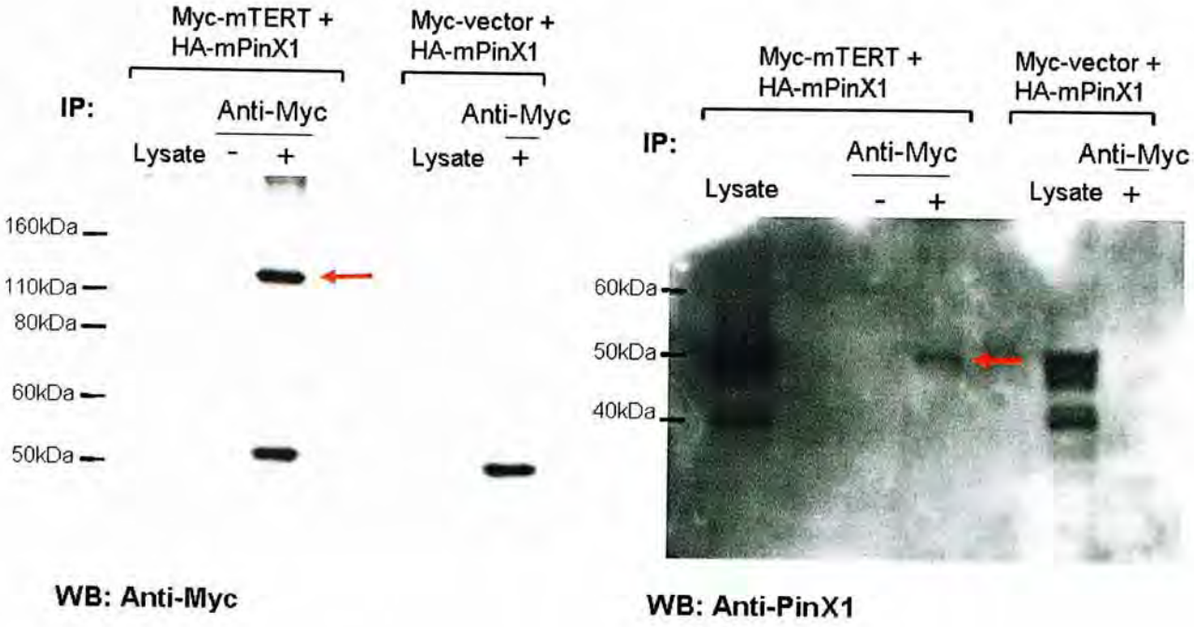


Figure 3.13 Representative blot of the Co-IP of Myc-mTERT and HA-mPinX1. Myc-mTERT was expressed in HEK cells and could be pulled down by anti-Myc antibody. (Left panel) The pull-down was specific as no specific band was detected in antibody negative control lane and pCMV-Myc vector lane. (Right panel) HA-mPinX1 could be found in the pull down lysate as detected by Anti-PinX1 antibody (as indicated by the red arrow). The interaction was specific to Myc-mTERT as no specific band was detected in antibody negative control lane and pCMV-Myc vector control lane.

Figure 3.14

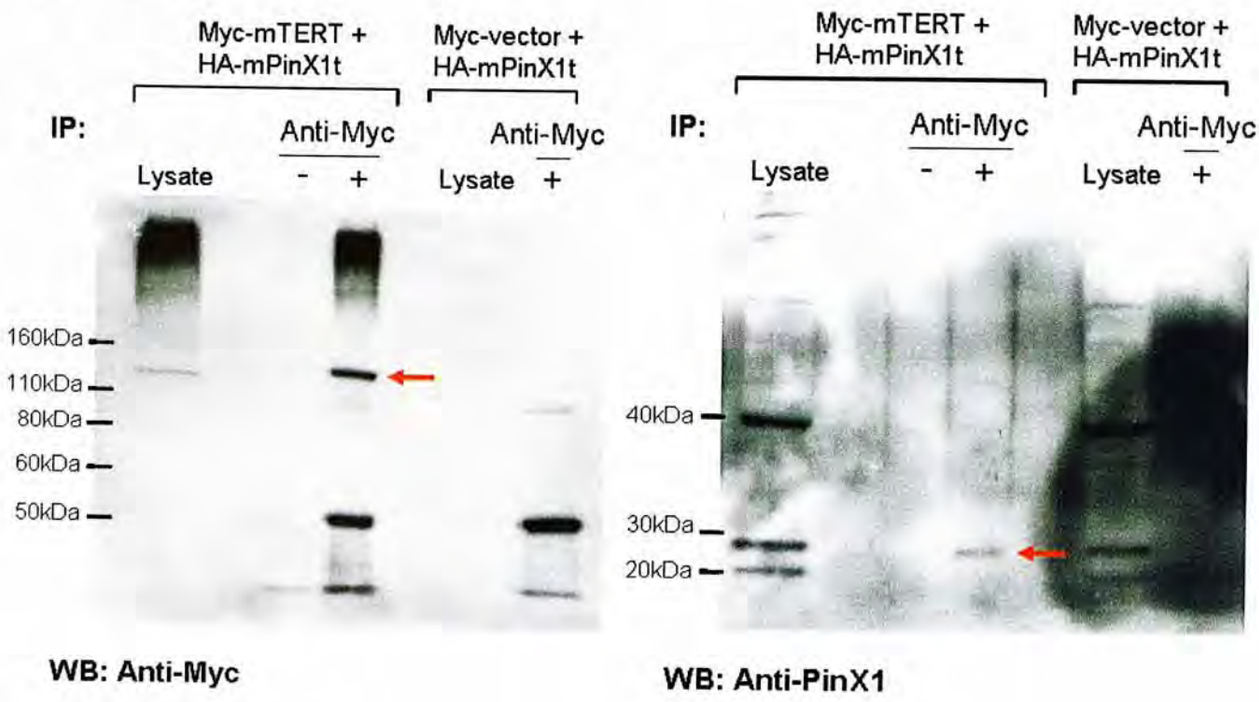


Figure 3.14 Representative blot of the Co-IP of Myc-mTERT and HA-mPinX1t. Myc-mTERT was expressed in HEK cells and could be pulled down by anti-Myc antibody. (Left panel) The pull-down was specific as no specific band was detected in antibody negative control lane and pCMV-Myc vector lane. (Right panel) HA-mPinX1t could be found in the pull down lysate as detected by Anti-PinX1 antibody (as indicated by the red arrow). The interaction was specific to Myc-mTERT as no specific band was detected in antibody negative control lane and pCMV-Myc vector control lane

3.6 Transient knockdown of mPinX1 in mESCs

To investigate the role of mPinX1 in maintaining the characteristics of mESCs, including proliferation, viability, pluripotency and cell cycle distribution, we performed knockdown experiments of mPinX1 by employing siRNAs against mPinX1. Since previous studies showed that PinX1 exerts its effect mainly by regulating telomerase activity [54, 112], the effect of mPinX1 knockdown on telomerase activity was also investigated.

The knockdown of mPinX1 was most significant at 24 hours after transfection (0.48 ± 0.06 Vs 1.00 ± 0.00 of the scrambled control, n=4, $p<0.05$, Fig.3.15), with a slight rebound at 48 hours (0.59 ± 0.11 vs 1.00 ± 0.00 of the scrambled control, n=4, $p<0.01$, Fig.3.15). On the other hand, mPinX1t was marginally knocked down at both time points (0.81 ± 0.07 Vs 1.00 ± 0.00 of the scrambled control at 24 hours, n=4, $p<0.001$; 0.83 ± 0.02 Vs 1.00 ± 0.00 of the scrambled control at 48 hours, n=4, $p<0.05$, Fig.3.15).

By western blot analysis, the protein of mPinX1 was also detected to be knocked down by siPinX1 at both 24hr and 48hr after transfection (0.74 ± 0.07 Vs 1.00 ± 0.00 of the scrambled control, n=6, $p<0.05$ at 24 hours, 0.83 ± 0.06 Vs 1.00 ± 0.00 of the scrambled control, n=4, $p<0.05$ at 48 hours, Fig.3.16).

3.6.1 Knockdown of mPinX1 decreased proliferation but did not change cell viability

Trypan blue exclusion assay was done at 24 and 48 hours after transfection. $5X10^4$ cells were plated in both groups one day prior to transfection. At 24 hours after transfection, the normalized number of viable cells in siPinX1 group was less than that of the scrambled siRNA group (0.89 ± 0.02 Vs 1.00 ± 0.00, n=4-5, $p<0.01$, Fig.3.17), meaning that the cell proliferation rate was decreased with mPinX1 knockdown. However, the effect faded out at 48 hours. On the other hand, there were no difference in cell viability between the two groups at both time points, showing that knockdown of mPinX1 did not change the viability (n=4-5, Fig.3.17).

3.6.2 Knockdown of mPinX1 decreased telomerase activity

TRAP assay was done at 24 hours and 48 hours after transfection. The telomerase activities of the siPinX1-transfected group were normalized to that of the scrambled siRNA-transfected group. siPinX1-transfected group showed a decreased telomerase activity at both time points (0.84 ± 0.04 Vs 1.00 ± 0.00 at 24 hours, n=4-5, $p<0.05$; 0.87 ± 0.04 Vs 1.00 ± 0.00 at 48 hours, n=4-5, $p<0.05$, Fig.3.18), meaning that knockdown of mPinX1 caused a decrease in telomerase activity. The results hint that mPinX1 did not act as a negative telomerase regulator at this cellular context.

3.6.3 Knockdown of mPinX1 did not change pluripotency

Western blot analysis was done at 24 hours and 48 hours after the transfection to investigate if knockdown of mPinX1 would affect pluripotency. Four pluripotent markers, Oct-4, Klf-4, Nanog and Sox-2, were chosen. β-Tubulin and H3 were used as the loading control. No significant change in their expressions could be detected after siPinX1 transfection at both time points, meaning that transient knockdown of mPinX1 did not change the pluripotent property of mESCs (n=3-4, Fig.3.19 & Fig.3.20).

3.6.4 Knockdown of mPinX1 did not affect cell cycle distribution

At 24 hours after transfection, cells were subjected to the investigation of the cell cycle distribution by flow cytometry. No significant change on cell cycle profile was detected after siPinX1 transfection, meaning that transient knockdown of mPinX1 did not affect the cell cycle distribution of mESCs (n=3, Fig.3.21).

Figure 3.15

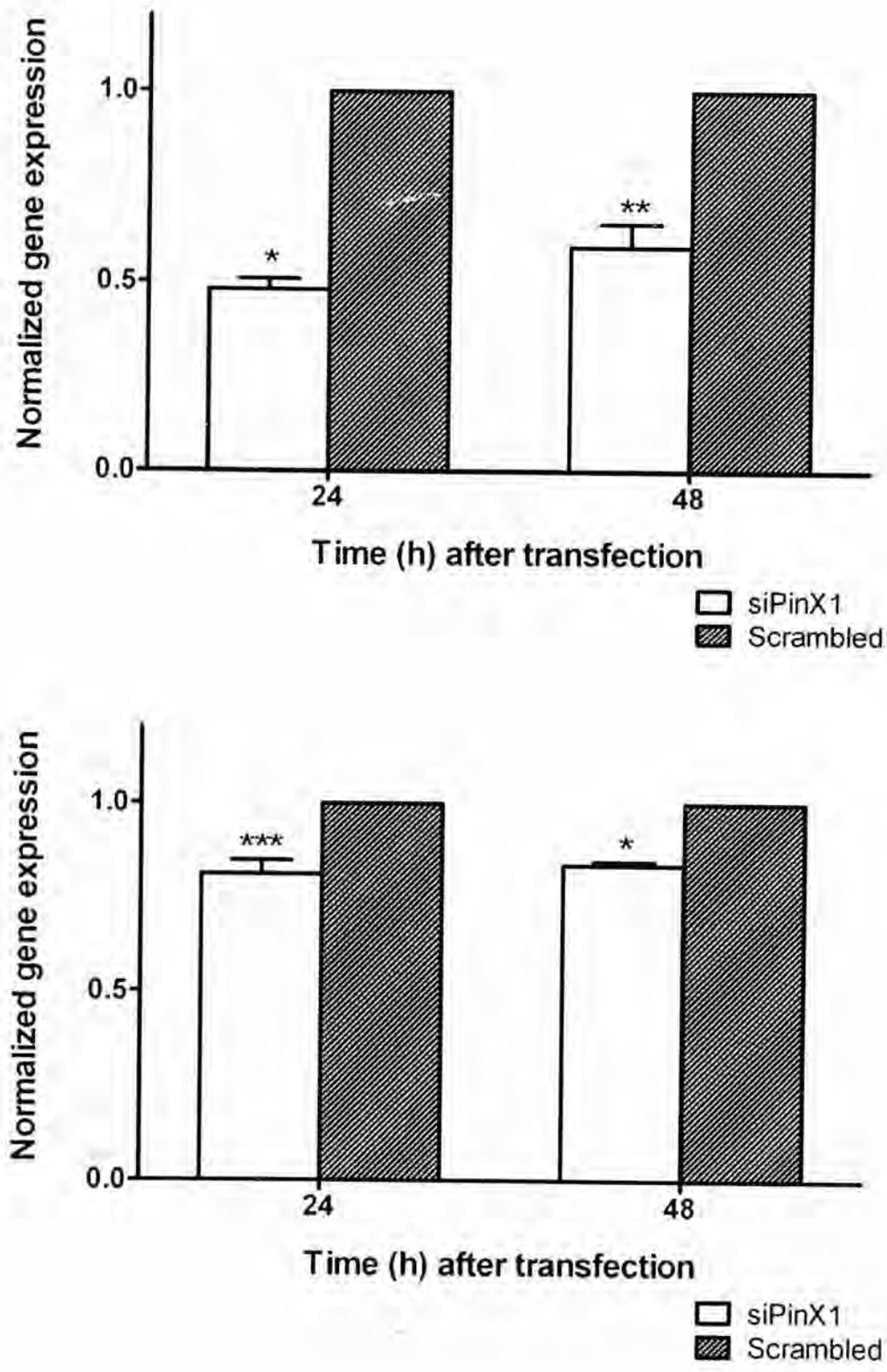


Figure 3.15 RT-qPCR results showing the expression of mPinX1 and mPinX1t after mPinX1 knockdown. The gene expression levels were normalized with that of scrambled siRNA transfected group. β-actin was used as the loading control. (Upper penal) The knockdown of mPinX1 was most significant at 24hr after transfection to ~0.5 fold, with a slight rebound at 48hr. (Lower penal) mPinX1t was knocked down to ~0.8 fold at both time points. Values are mean ± S.E.M. of 4 experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$ Vs Scrambled siRNA group.

Figure 3.16

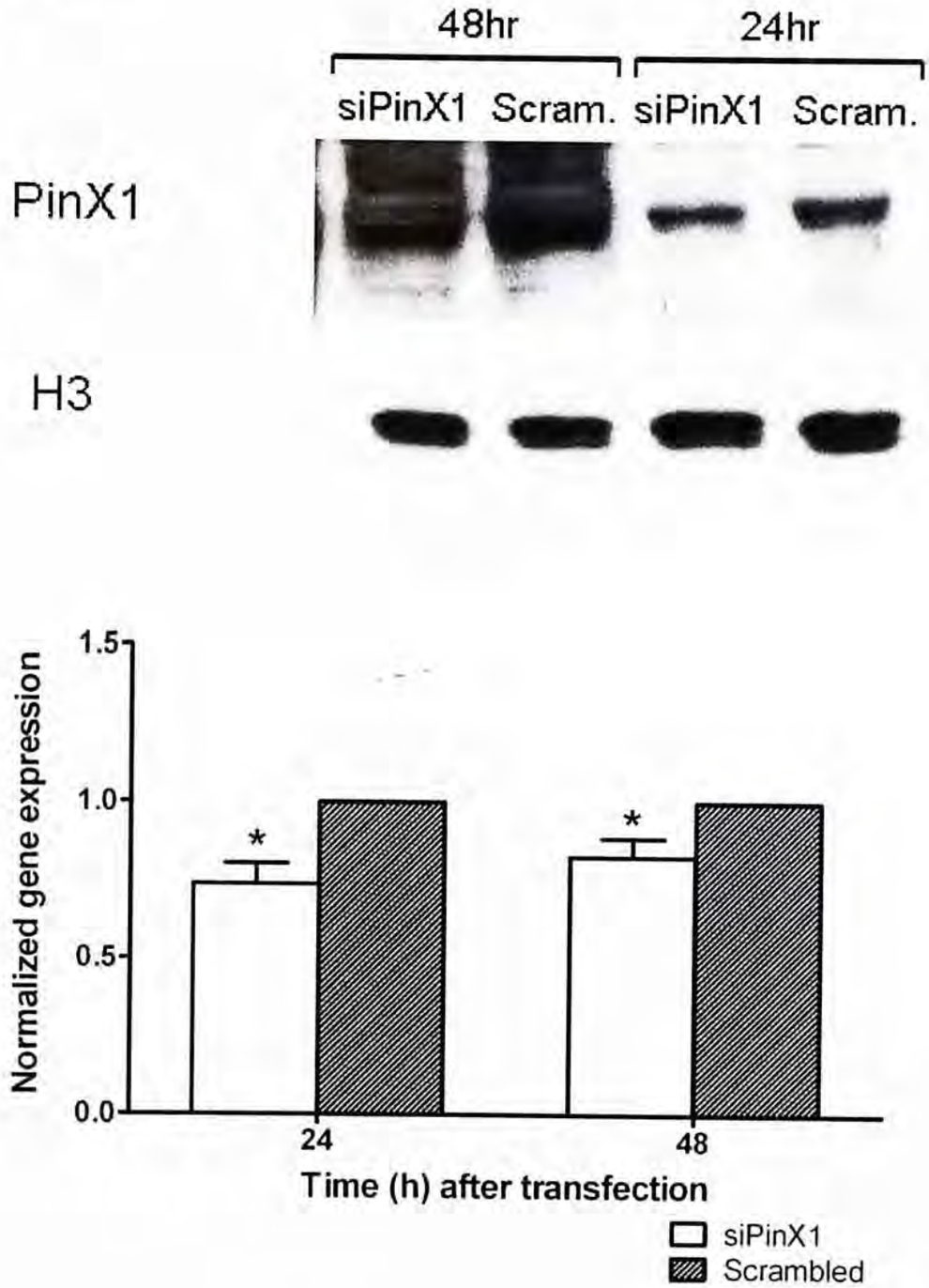


Figure 3.16 Representative western blot and bar chart showing mPinX1 protein level after mPinX1 knockdown. mPinX1 protein was knocked down to ~0.7-0.8 fold at both 24hr and 48hr after transfection. Values are mean ± S.E.M. of 4-6 experiments. *$p<0.05$ Vs Scrambled siRNA group.

Figure 3.17

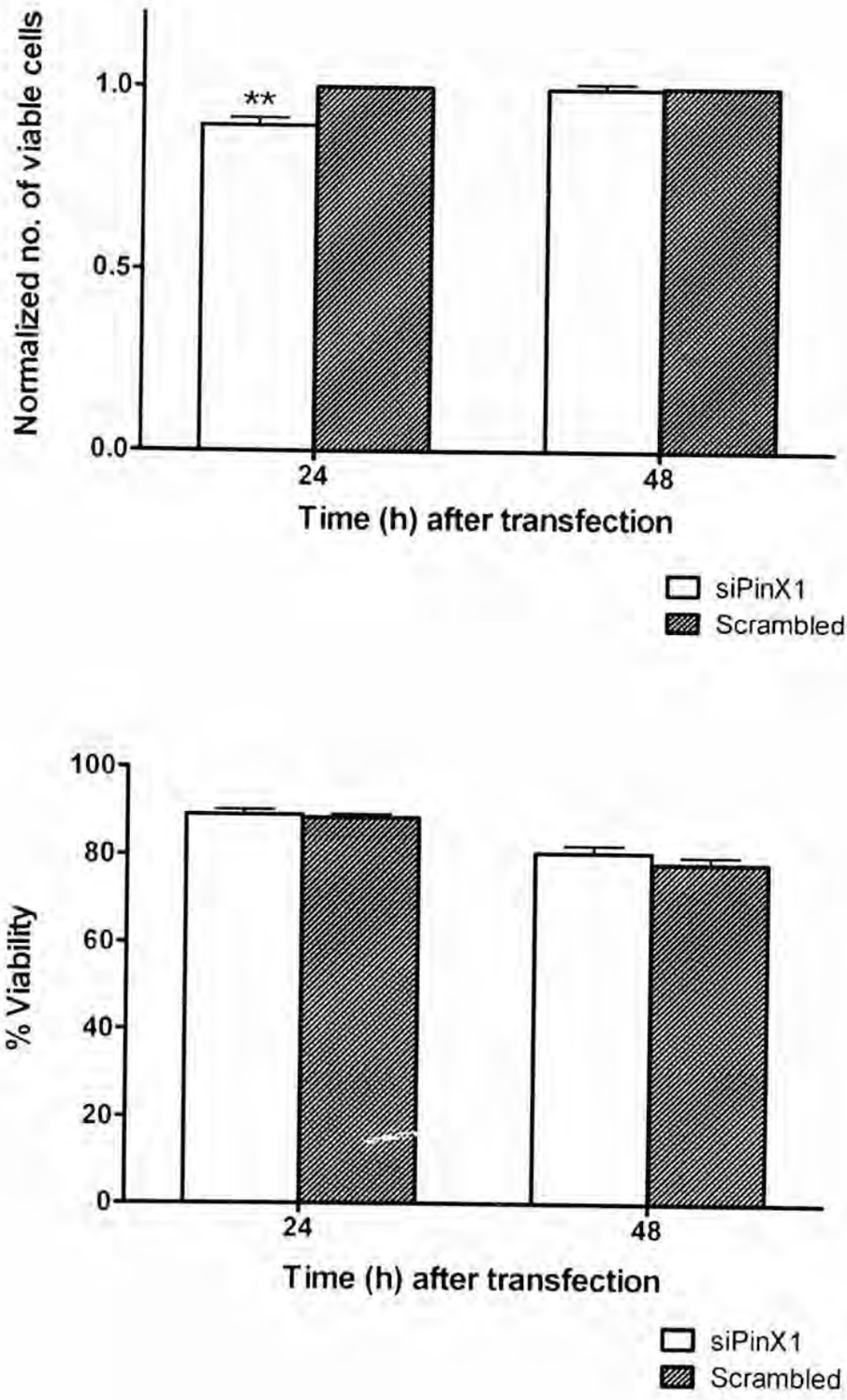


Figure 3.17 Trypan blue exclusion assay results after mPinX1 knockdown. (Upper panel) At 24hr after transfection, the number of viable cells in siPinX1 group was ~10% less than the scrambled siRNA group, but the effect faded out at 48hr. (Lower panel) There was no difference in viability between the two groups at both time points. Values are mean ± S.E.M. of 4-5 experiments. **$p<0.01$ Vs Scrambled siRNA group.

Figure 3.18

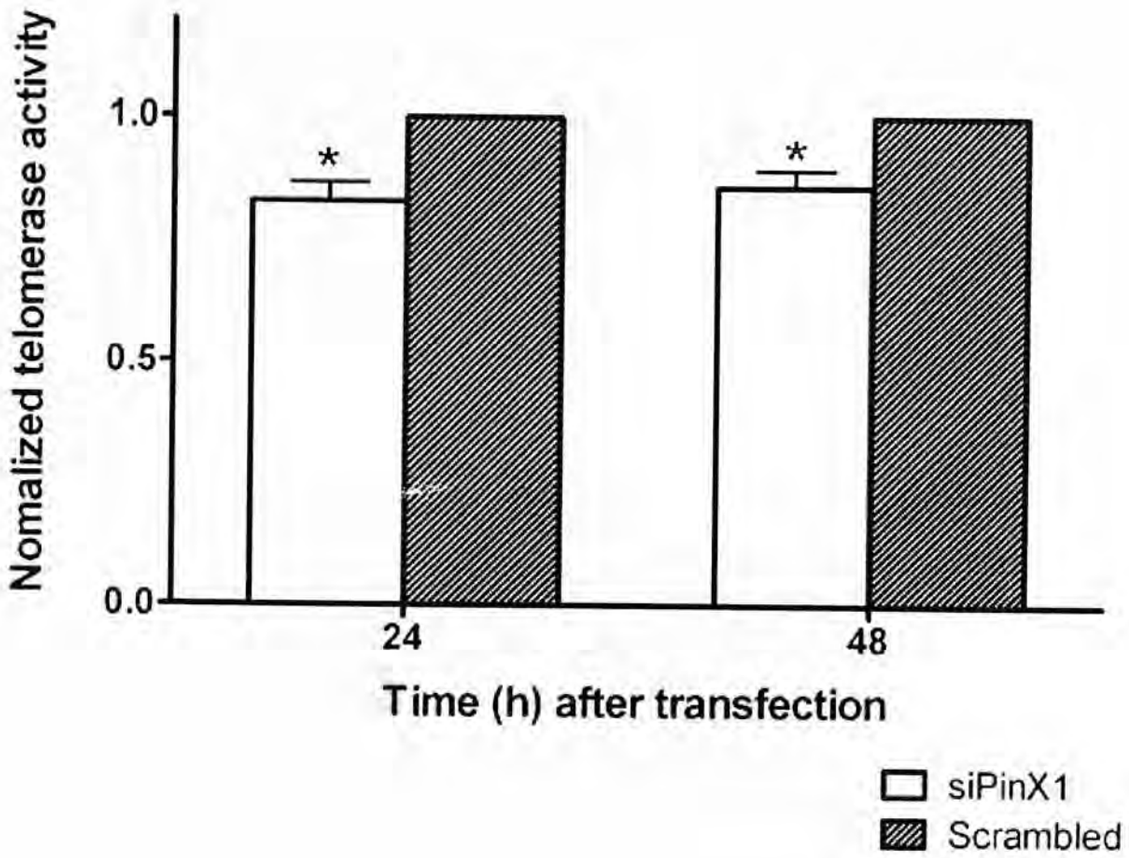


Figure 3.18 TRAP assay result showing the telomerase activity after mPinX1 knockdown. The telomerase activities were normalized with the scrambled siRNA transfected group. siPinX1 transfected group showed ~10% decreased telomerase activity at both time points. Values are mean ± S.E.M. of 4-5 experiments. *$p<0.05$ Vs Scrambled siRNA group.

Figure 3.19

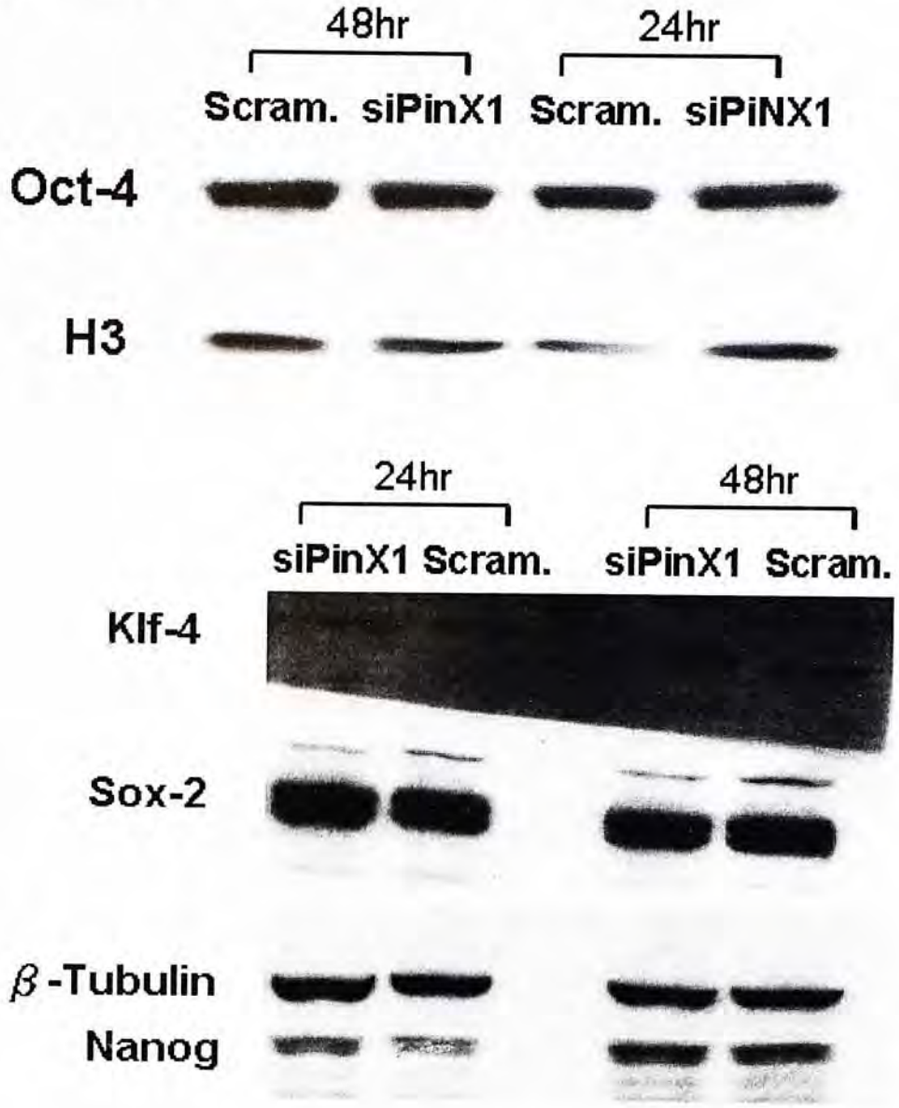


Figure 3.19 Representative western blot showing the expression of 4 pluripotent markers Oct-4, Klf-4, Sox-2 and Nanog after mPinX1 knockdown. H3 and β-Tubulin were used as the loading controls.

Figure 3.20

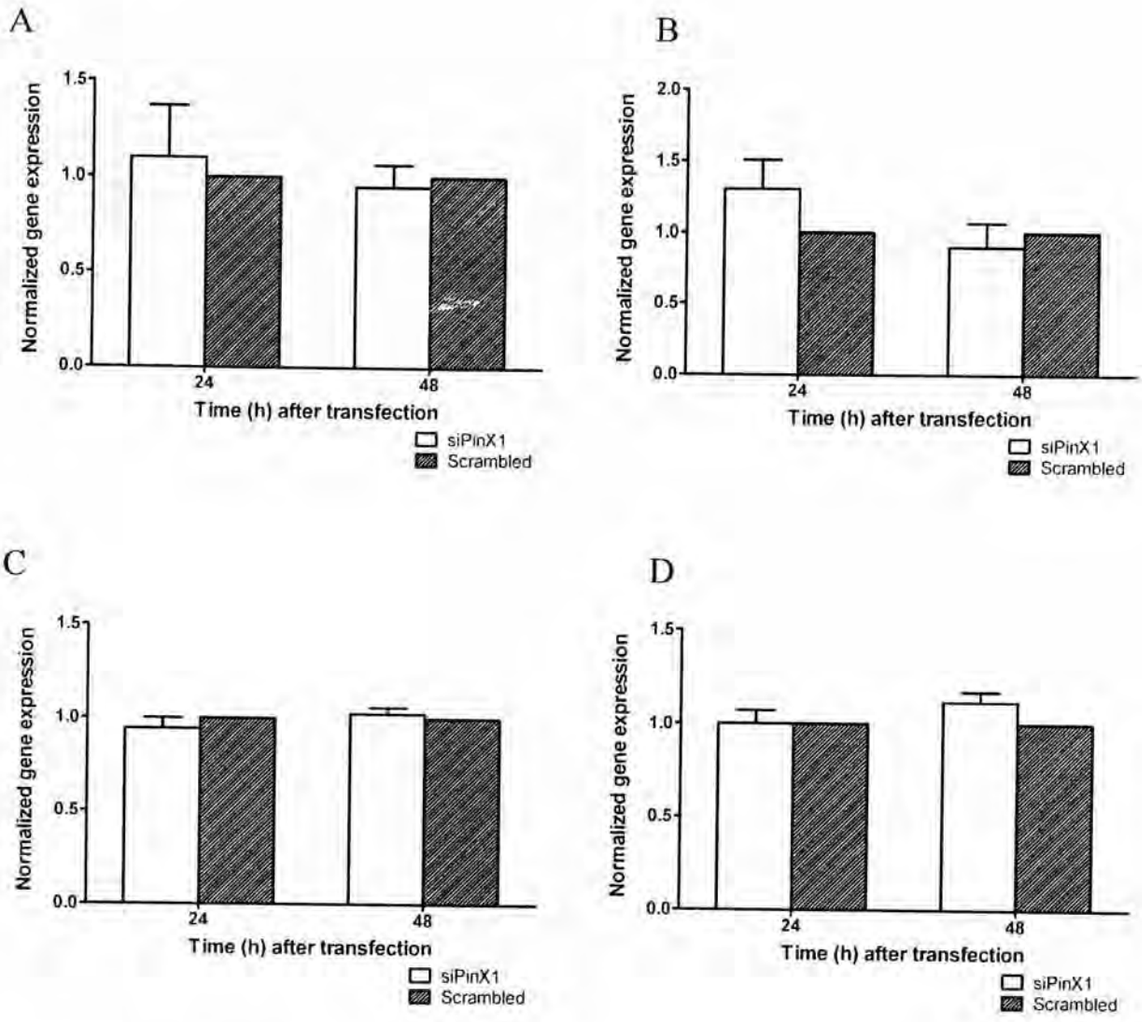


Figure 3.20 Bar charts showing the expression of 4 pluripotent markers (A) Oct-4, (B) Klf-4, (C) Sox-2 and (D) Nanog after mPinX1 knockdown. There was no significant change in their expressions after siPinX1 transfection at both time points. Values are mean ± S.E.M. of 3 experiments.

Figure 3.21

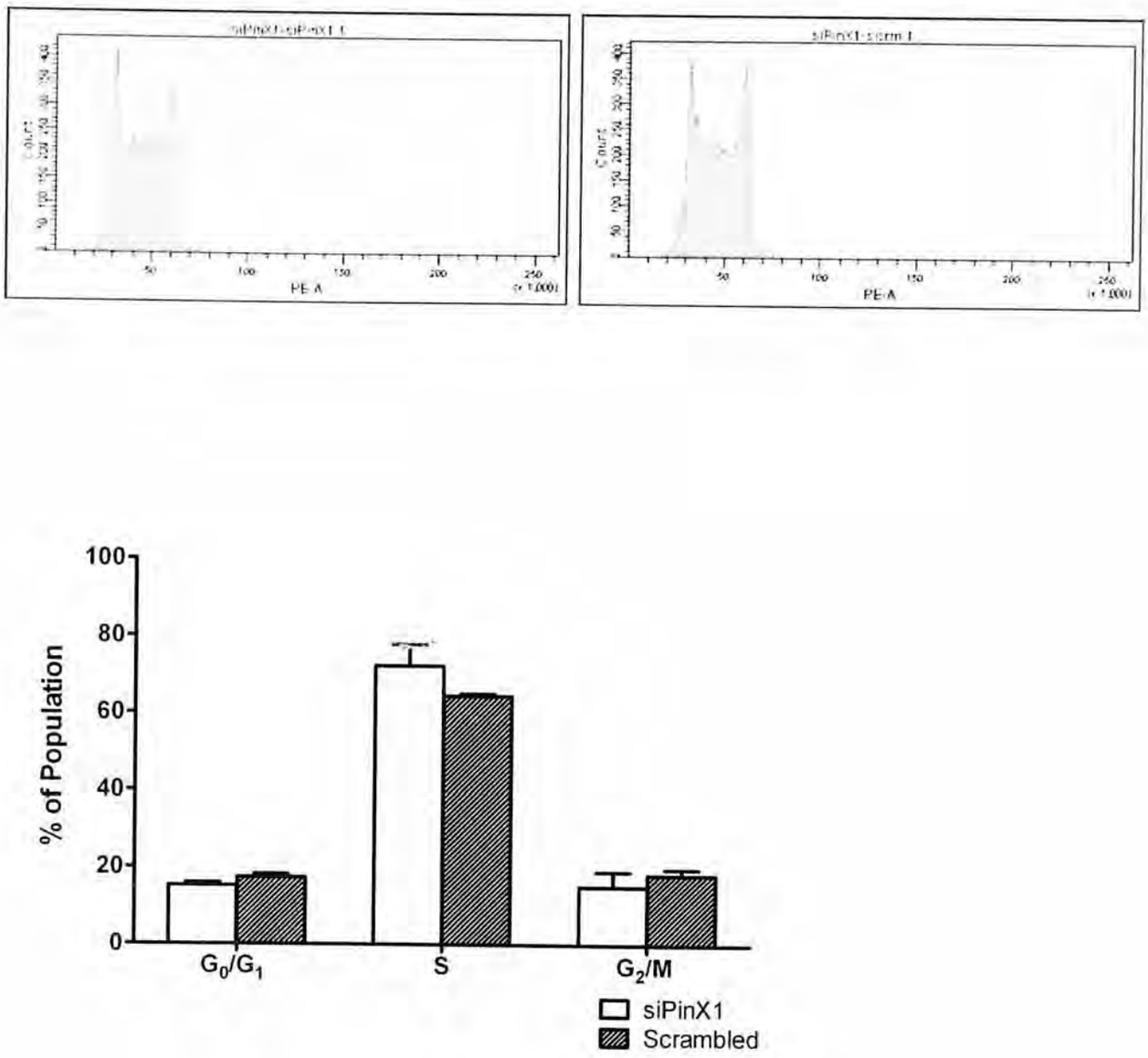


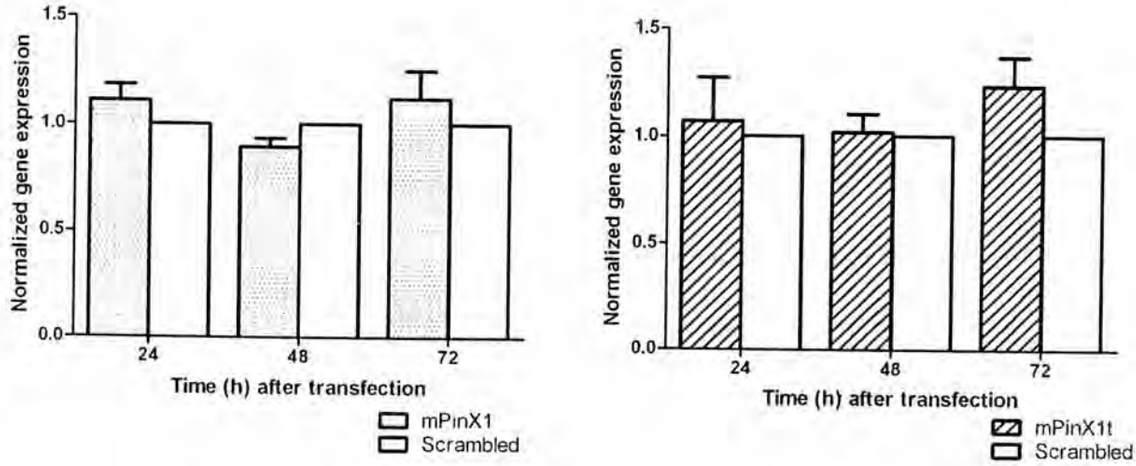
Figure 3.21 The cell cycle profile of (upper left panel) the siPinX1 tranfected and (upper right panel) the scrambled siRNA transfected mESCs at 24hr after tranfection and (lower panel) the bar chart showing the % of population in each cell cycle phases. There was no significant change in the % of population in the three cell cycle phases after mPinX1 knockdown. Values are mean ± S.E.M. of 3 experiments.

3.7 Transient knockdown of mPinX1t using siRNA against mPinX1t in mESCs

In order to investigate the role of mPinX1t in mESCs, we designed 2 siRNAs targeting 2 different specific regions on mPinX1t and hoped to see the knock down effect of mPinX1t in mESCs. However, both siRNAs failed to knockdown mPinX1t gene (n=3, Fig.3.22).

Figure 3.22

siRNA 1



siRNA 2

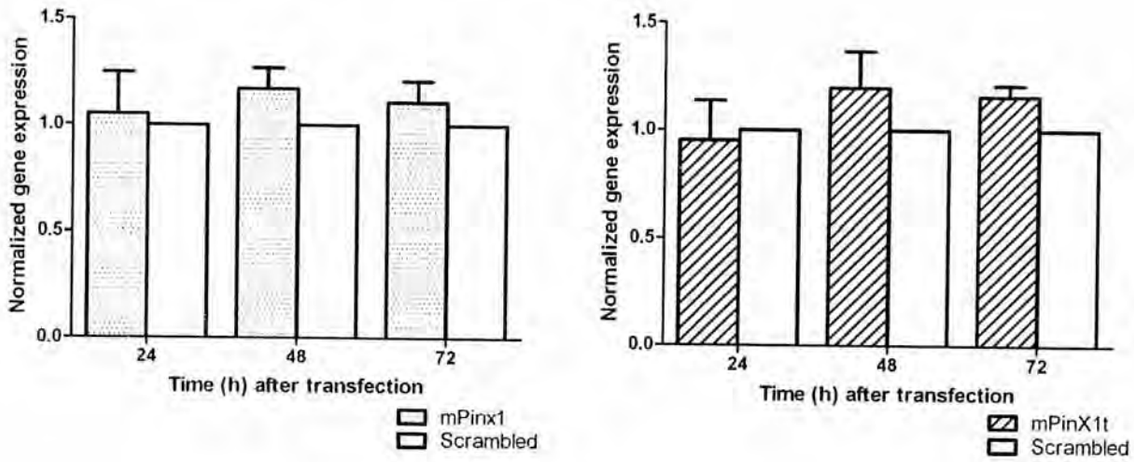


Figure 3.22 RT-qPCR results showing the expression of mPinX1 and mPinX1t in mESCs by 2 siRNAs targeting 2 different regions on mPinX1t. The gene expression levels were normalized with scrambled siRNA transfected group. β-actin was used as the loading control. Both siRNAs failed to knock-down mPinX1t at mRNA level. Values are mean ± S.E.M. of 3 experiments.

3.8 Transient over-expression of mPinX1 and mPinX1t in mESCs

Both mPinX1 and mPinX1t were subcloned into pCMV-Myc vector, and then the Myc-mPinX1 and Myc-mPinX1t were further subcloned into the pWPI vector (Fig. 3.23). pWPI vector contains the EF-1α promoter which can allow genes under its control to be robustly expressed in mESCs [95]. The resultant pWPI-Myc-mPinX1 (namely pWPI-mPinX1 in the following parts) and pWPI-Myc-mPinX1t (namely pWPI-mPinX1t in the following parts) plasmids were then transfected into mESCs, followed by proliferation assay, viability assay, TRAP and western blot analysis of the pluripotent markers.

In pWPI-mPinX1-transfected group, over-expression of mPinX1 was most significant at 24 hours after transfection where mPinX1 expression was increased to 24.16 ± 4.14 fold when compared with that of empty vector-transfected group (n=4, $p<0.05$, Fig.3.24). At 48 hours and 72 hours after transfection, the over-expression effect was diminished to 3.65 ± 0.32 (n=4, $p<0.01$, Fig.3.24) and 2.25 ± 0.16 fold of increase, respectively (n=3, $p<0.01$, Fig.3.24). The expression of mPinX1t gene was not affected when mPinX1 was over-expressed at both 24hr, 48hr and 72hr (n=3-6, Fig.3.24).

On the other hand, in pWPI-mPinX1t-transfected group, over-expression of the mPinX1t was most significant at 24 hours after transfection where mPinX1t

expression was increased to 657.07 ± 38.17 fold when compared with that of empty vector-transfected group (n=4, $p<0.001$, Fig.3.25). At 48 hours and 72 hours after transfection, the over-expression effect was diminished to 43.97 ± 10.72 and 11.81 ± 3.17 (n=3, Fig.3.25) fold of increase, respectively. Interestingly, when mPinX1t was over-expressed, the expression of mPinX1 also changed significantly, with 11.77 ± 0.77 fold of increase at 24 hours (n=4, $p<0.001$, Fig.3.25). The increase was not significant at 48 and 72 hours (n=3, Fig.3.25). This increase suggested a possible regulatory role of mPinX1t on mPinX1 gene.

By western blotting, the overexpression of mPinX1 and mPinX1t could be detected at protein level at 24 hours after transfection, as shown by the appearance of the additional band of Myc-mPinX1 and Myc-mPinX1t in the respective over-expression groups. However, at 48 hours after transfection, only the Myc-mPinX1 could be detected (n=2, Fig.3.26).

3.8.1 Over-expression of mPinX1 and mPinX1t decreased cell proliferation but did not affect cell viability

Trypan blue exclusion assay was done at 24, 48 and 72 hours after transfection. $5X10^4$ cells were plated one day prior to transfection. For pWPI-mPinX1-transfected group, the normalized number of viable cells was less than that of the empty vector

group at all time points (0.88 ± 0.02 Vs 1.00 ± 0.00 at 24 hours, n=5, $p<0.01$; 0.87 ± 0.03 Vs 1.00 ± 0.00 at 48 hours, n=6, $p<0.01$; 0.86 ± 0.02 Vs 1.00 ± 0.00 at 72 hours, n=3, $p<0.05$, Fig.3.27), meaning that the cell proliferation rate was decreased with mPinX1 over-expression. For pWPI-mPinX1t-transfected group, the number of viable cells was also significantly less than that of the empty vector group at 24 hours and 72 hours after transfection (0.87±0.04 Vs 1±0.00 at 24 hours, n=5, $p<0.05$; 0.89±0.02 Vs 1±0.00 at 72 hours, n=3, $p<0.05$, Fig.3.27), meaning that mPinX1t over-expression decreased the proliferation of mESCs. There was no significant difference in cell viability between the two groups and the empty vector group at all time points, suggesting that the over-expression of mPinX1 and mPinX1t did not change the cell viability (n=5-6, Fig.3.27).

3.8.2 Over-expression of mPinX1 increased telomerase activity

TRAP assay was done at 24, 48 and 72 hours after transfection. The telomerase activities of the overexpression group were normalized to that of the empty vector group. pWPI-mPinX1-transfected group showed an increased telomerase activity 24 hours after transfection when compared to that of the empty vector-transfected group (1.10 ± 0.03 Vs 1.00 ± 0.00 at 24 hours, n=5, $p<0.05$, Fig.3.28), suggesting that over-expression of mPinX1 caused an increase in telomerase activity. The results hint

that mPinX1 did not act as a negative telomerase regulator at this cellular context. On the other hand, over-expression of mPinX1t did not change the telomerase activity (n=3-5, Fig.3.28).

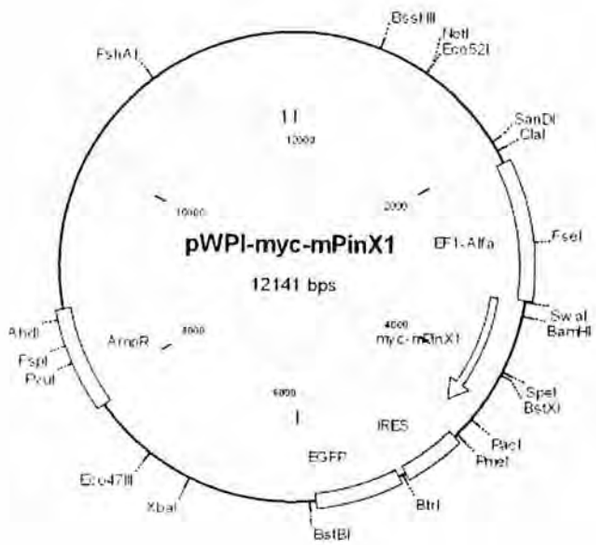
3.8.3 Over-expression of mPinX1 and mPinX1t did not affect pluripotency

Western blot was done at 24hr and 48hr after transfection to investigate if over-expression of mPinX1 and mPinX1t would affect pluripotency. Pluripotent markers Oct-4, Klf-4, Sox-2 and Nanog were chosen; β-Tubulin was used as the loading control. From the results, there was no significant change in their expressions after pWPI-mPinX1 and pWPI-mPinX1t transfection at all time points, meaning that over-expression of both genes did not change the pluripotent property of the cells (n=3, Fig.3.29 & Fig.3.30).

3.8.4 Over-expression of mPinX1 and mPinX1t did not affect cell cycle distribution

At 24 hours after transfection, cells were subjected to flow cytometry to investigate the cell cycle distribution. No significant change was detected after pWPI-mPinX1 or pWPI-mPinX1t transfection, meaning that transient over-expression of mPinX1 or mPinX1t did not affect the cell cycle progression of mESCs (n=2-3, Fig.3.31).

Figure 3.23



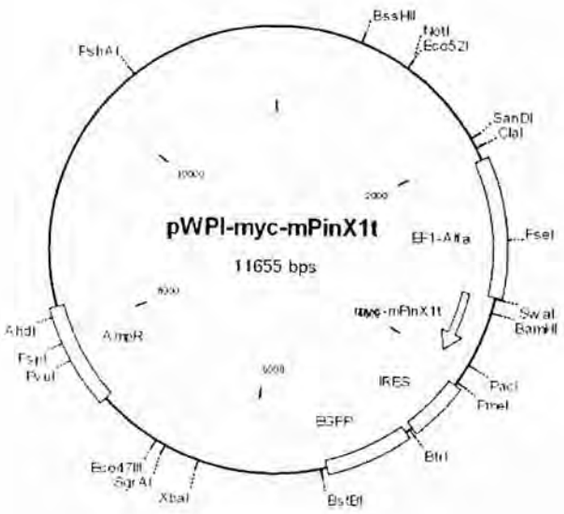


Figure 3.23 The vector maps of (upper panel) pWPI-Myc-mPinX1 and (lower panel) pWPI-Myc-mPinX1t.

Figure 3.24

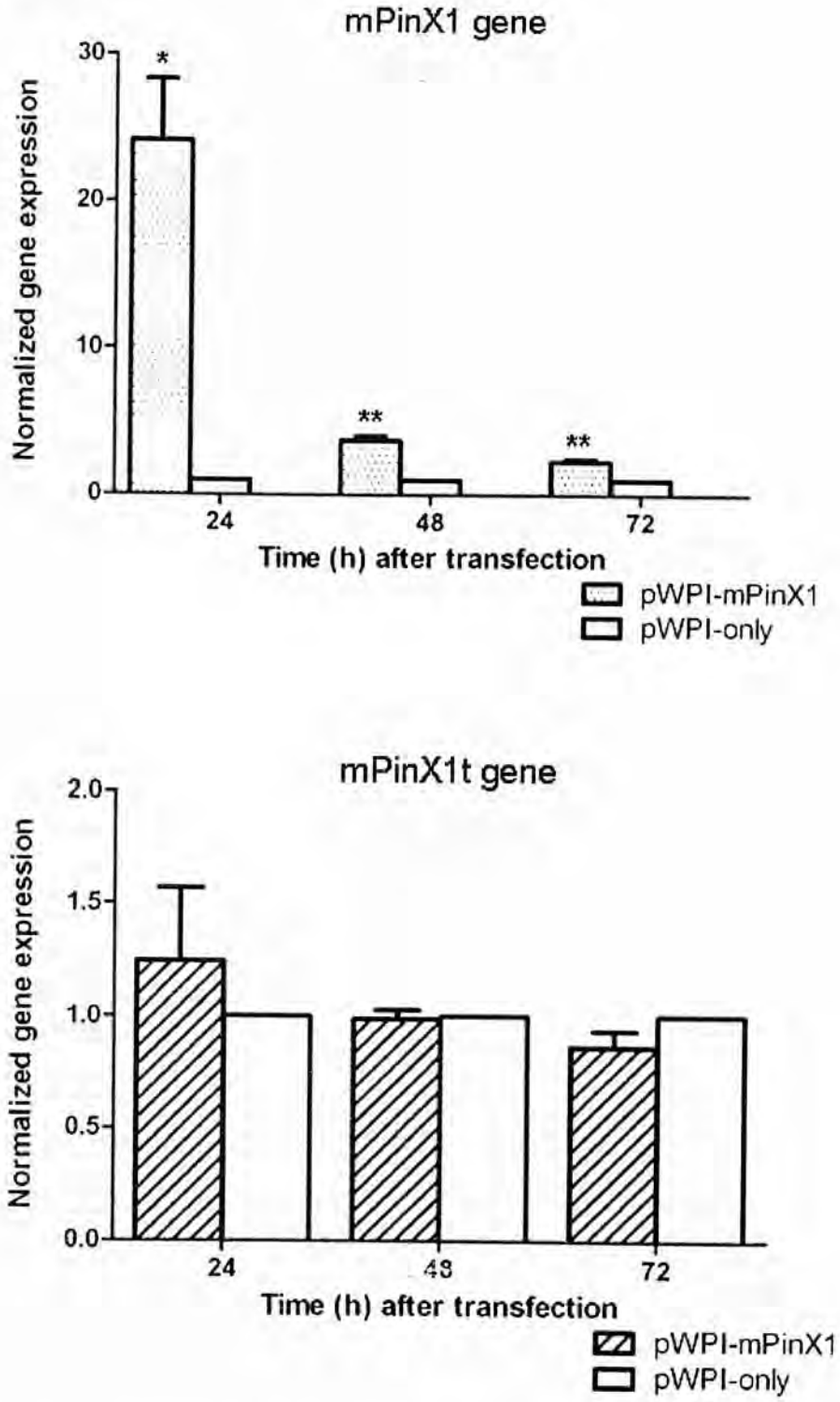


Figure 3.24 RT-qPCR results showing the expression of (upper panel) mPinX1 and (lower panel) mPinX1t of pWPI-mPinX1 and control pWPI only group. The gene expressions were normalized with that of empty vector group. β-actin was used as the loading control. (Upper panel) The over-expression of mPinX1 was most significant at 24hr after transfection where mPinX1 expression was increased for ~20 fold. At 48hr and 72hr the effect was diminished to ~2-3 fold of increase. (Lower panel) The expression of mPinX1t gene did not change significantly during the process. Values are mean ± S.E.M. of 3-6 experiments. *$p<0.05$, **$p<0.01$ Vs pWPI-only group.

Figure 3.25

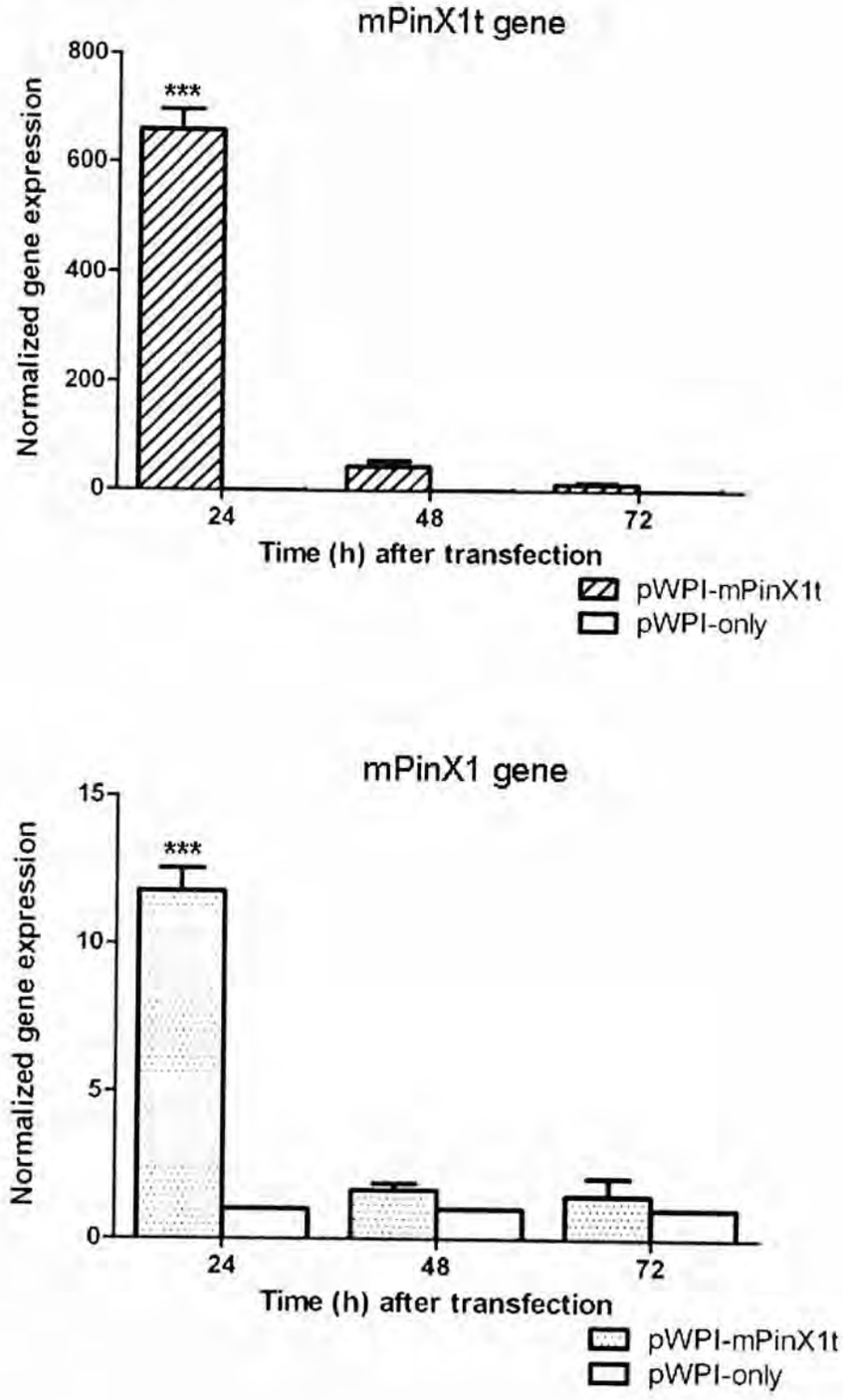


Figure 3.25 RT-qPCR results showing the expression of (upper panel) mPinX1t and (lower panel) mPinX1 of pWPI-mPinX1t and control pWPI only group. The gene expression levels were normalized with that of control group. β-actin was used as the loading control. (Upper panel) The over-experssion of mPinX1t was most significant at 24hr after transfection where mPinX1t expression was increased for around 600 fold. At 48hr and 72hr, the effect was diminished. (Lower panel) The expressions of mPinX1 gene also increased significantly to around 10 fold at 24hr. Values are mean ± S.E.M. of 3-6 experiments. ***$p<0.001$ Vs pWPI-only group.

Figure 3.26

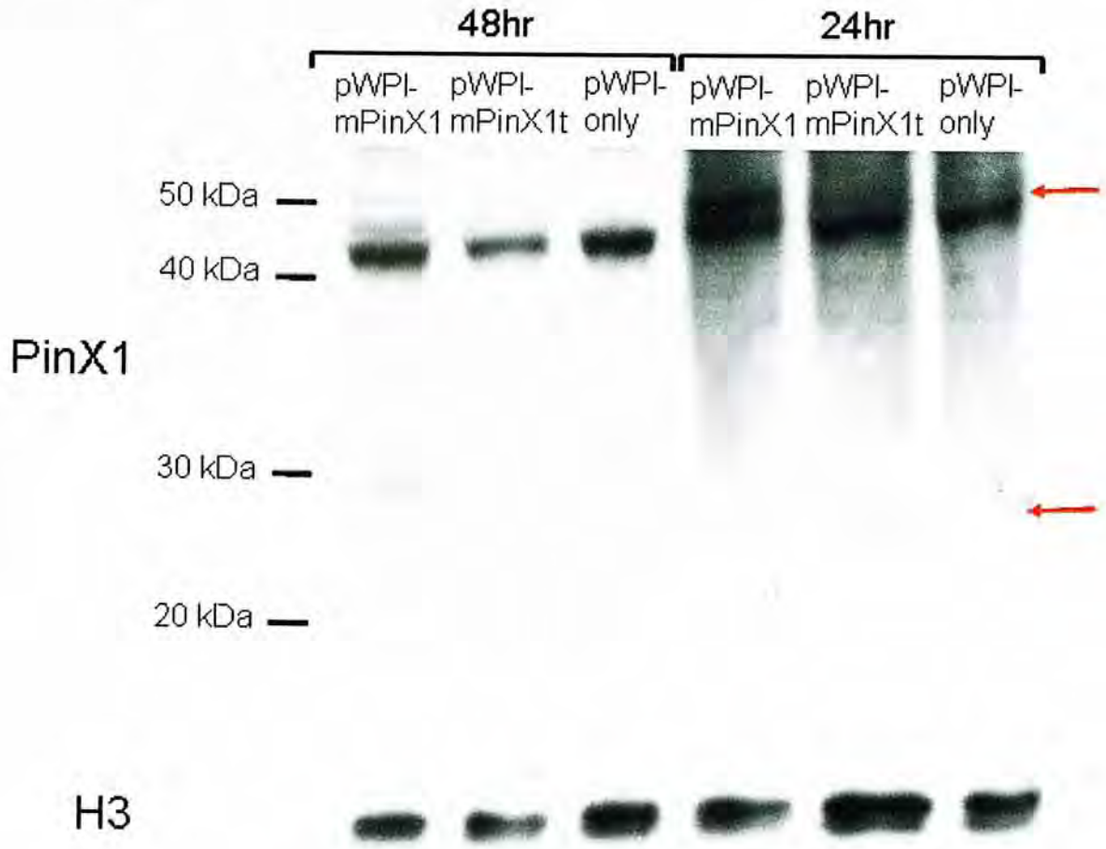


Figure 3.26 Representative western blot of mPinX1 and mPinX1t at protein level of pWPI-mPinX1, pWPI-mPinX1t and pWPI-only group. H3 was used as the loading control. The additional Myc-mPinX1 band (upper red arrow) and Myc-mPinX1t band (lower red arrow) could be detected at 24hr after transfection of pWPI-mPinX1 and pWPI-mPinX1t respectively, but only the Myc-mPinX1 could be detected at 48hr after transfection of pWPI-mPinX1.

Figure 3.27

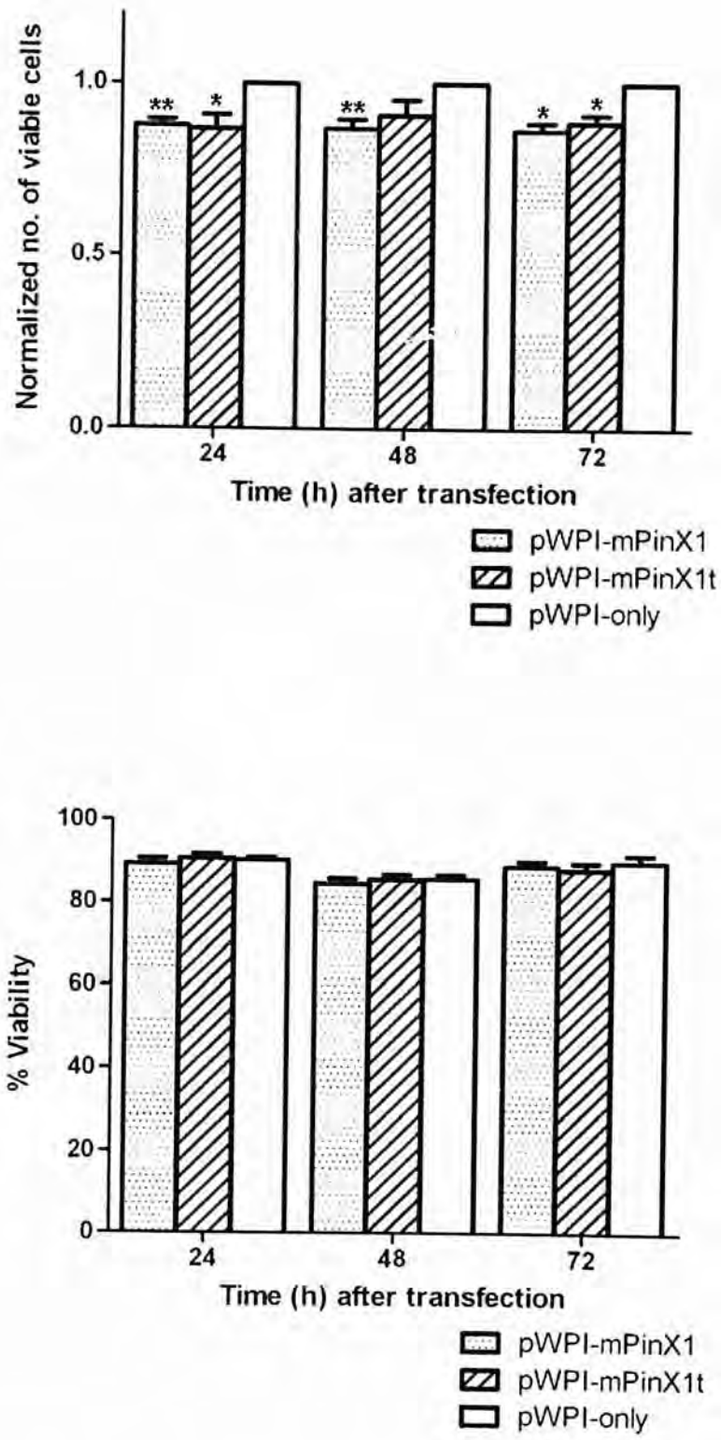


Figure 3.27 Trypan blue exclusion assay results at 24hr, 48hr and 72hr after transfection. (Upper panel) For pWPI-mPinX1 group, the number of viable cells was ~10% less than that of the empty vector group at all time points. For pWPI-mPinX1t group, the number of viable cells was also shown ~10% decrease compared to that of the empty vector group at 24hr and 72hr after transfection. (Lower panel) There was no significant difference in % of viability between the two groups and that of the empty vector group at all time points. Values are mean ± S.E.M. of 3-6 experiments. *$p<0.05$, **$p<0.01$ Vs pWPI-only group.

Figure 3.28

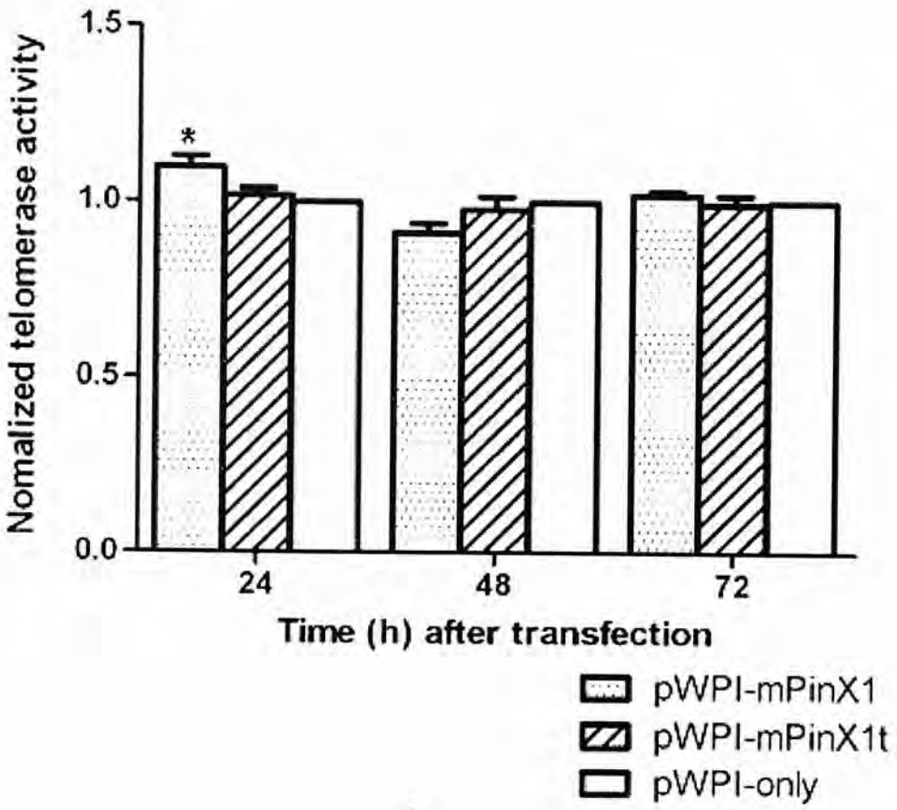


Figure 3.28 TRAP assay result showing the telomerase activity at 24hr, 48hr and 72hr after transfection. The telomerase activities were normalized with that of the pWPI empty vector group. pWPI-mPinX1 group showed a ~10% increased telomerase activity at 24hr. Values are mean ± S.E.M. of 3-5 experiments. *$p<0.05$ Vs pWPI-only group.

Figure 3.29

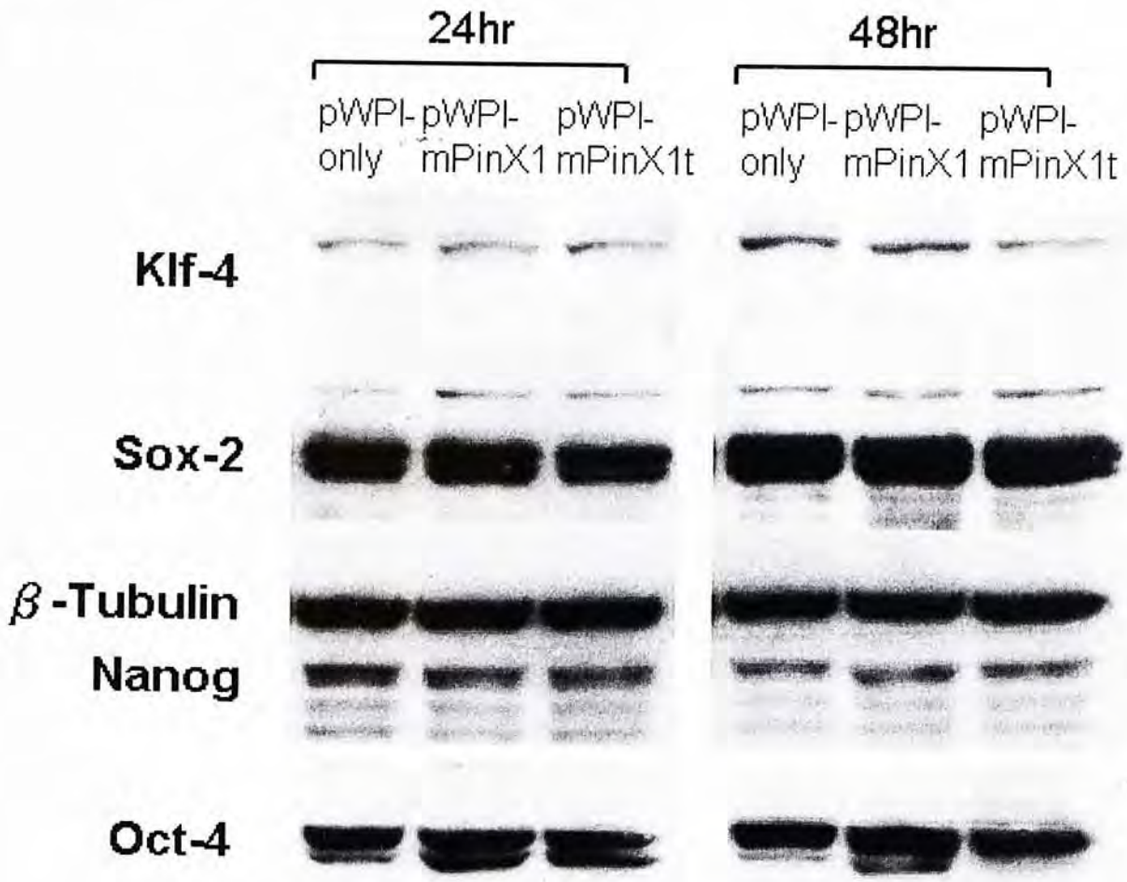


Figure 3.29 Representative western blot results showing the expression of 4 pluripotent markers Oct-4, Klf-4, Sox-2 and Nanog at 24hr and 48hr after transfection of pWPI-mPinX1, pWPI-mPinX1t, pWPI-only. β-Tubulin was used as the loading control.

Figure 3.30

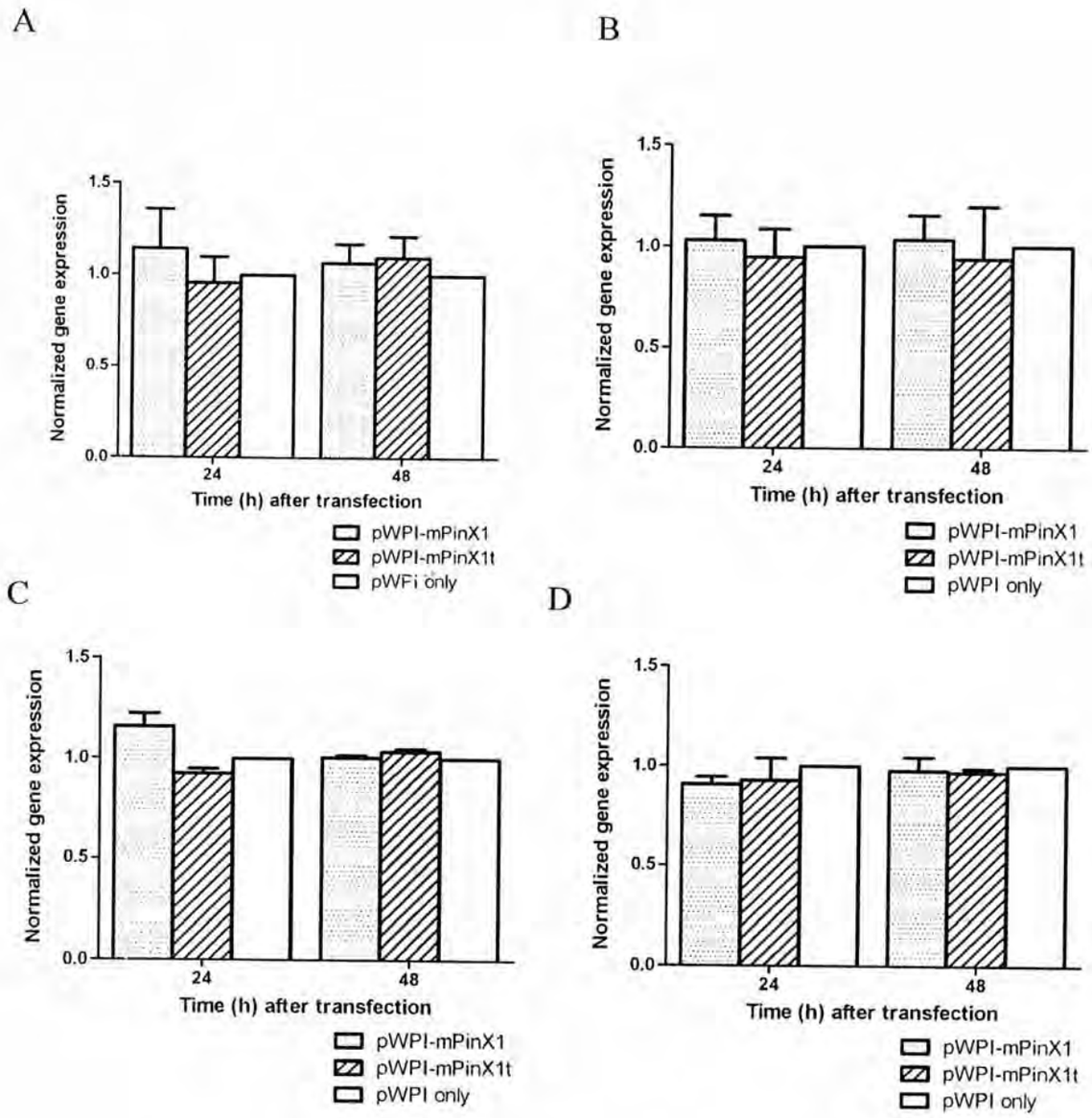
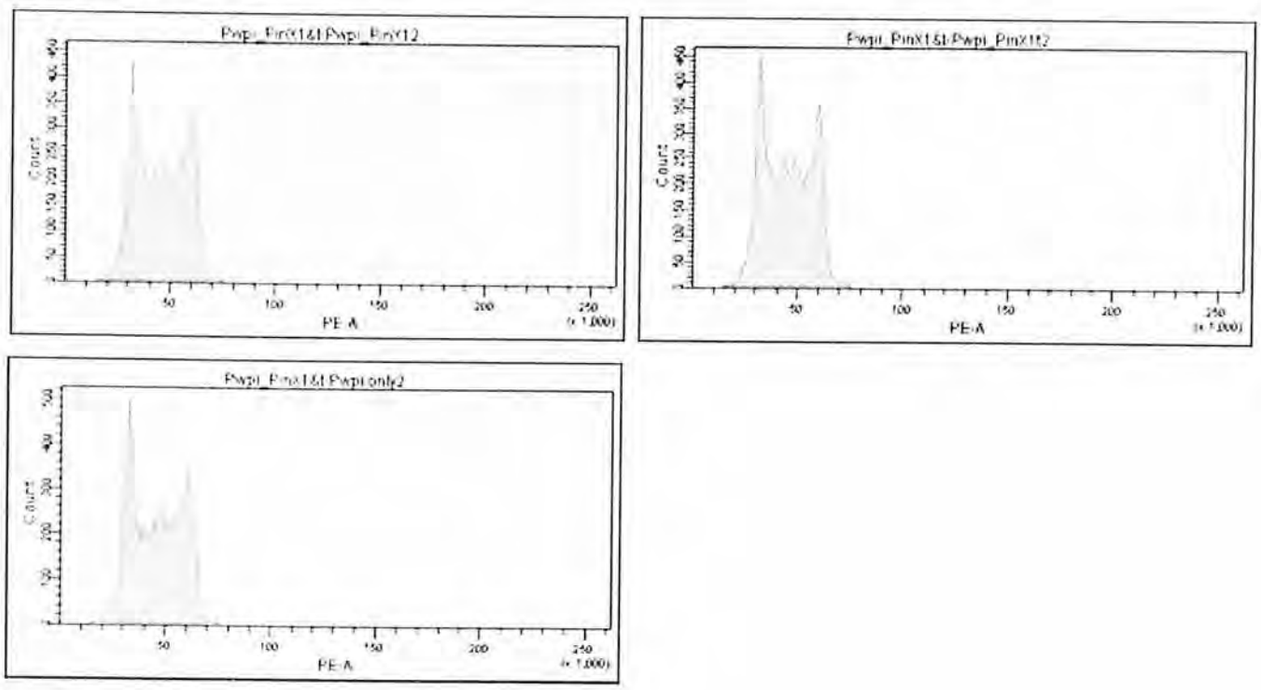


Figure 3.30 Bar charts showing the expression levels of 4 pluripotent markers (A) Oct-4, (B) Klf-4, (C) Sox-2 and (D) Nanog at 24hr and 48hr after transfection of pWPI-mPinX1, pWPI-mPinX1t and pWPI-only. There was no significant change in their expression levels at both time points. Values are mean ± S.E.M. of 3 experiments.

Figure 3.31



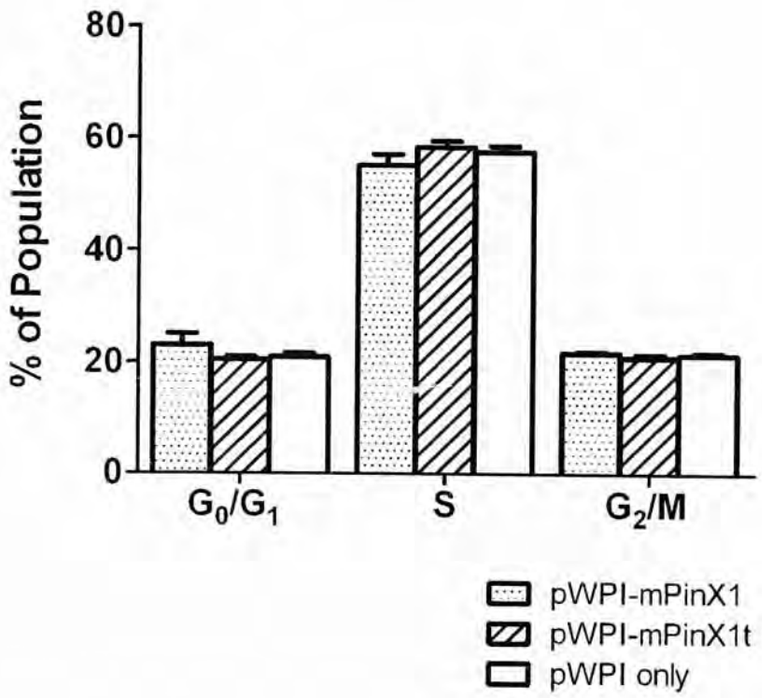


Figure 3.31 The cell cycle profiles of (upper left panel) the pWPI-mPinX1 transfected, (upper right panel) pWPI-mPinX1t transfected and (middle panel) pWPI-only transfected mESCs at 24hr after transfection and (lower panel) the bar chart showing the % of population in each cell cycle phases. There was no significant change in the % of population in the three cell cycle phases after pWPI-mPinX1 and pWPI-mPinX1t transfection. Values are mean ± S.E.M. of 2-3 experiments.

3.9 Stable over-expression and knockdown of mPinX1 and mPinX1t in mESCs

Stable over-expression and knockdown of the two genes were done to investigate if changing the expression of these 2 genes in the long-run would exert any cellular effect in a way similar to (yet with amplified effect) the results observed in transient experiments. The three plasmids used in the previous transient over-expression experiments, namely the pWPI-mPinX1, pWPI-mPinX1t and pWPI vectors, were used for the stable over-expression experiment. pLVTHM-shPinX1 and the pLVTHM-shScrambled plasmids were also generated (Fig.3.32). All the plasmids were introduced into mESCs by lenti-viral infection and stable clones were selected (as shown in Chapter 2 Materials and Methods). For mPinX1 over-expression group, the four clones selected were named as Pwpi-PinX1 C1'A, C13, C1B and C1'B. For mPinX1t over-expression group, the three selected were named as Pwpi-PinX1t C3A, C8B and C9A. For over-expression control group, the three clones selected were named as Pwpi-only C10' C11' and C12'. (Fig.3.33) For mPinX1 knockdown group, the three clones were named as shPinX1 C5, C1B and C1'B. For the knockdown control group, the three clones were named as shScrambled C3, C7 and C8. (Fig.3.34) The selected clones were established to stable cell lines and the effects on proliferation, viability, telomerase activity, pluripotency were examined.

Figure 3.32

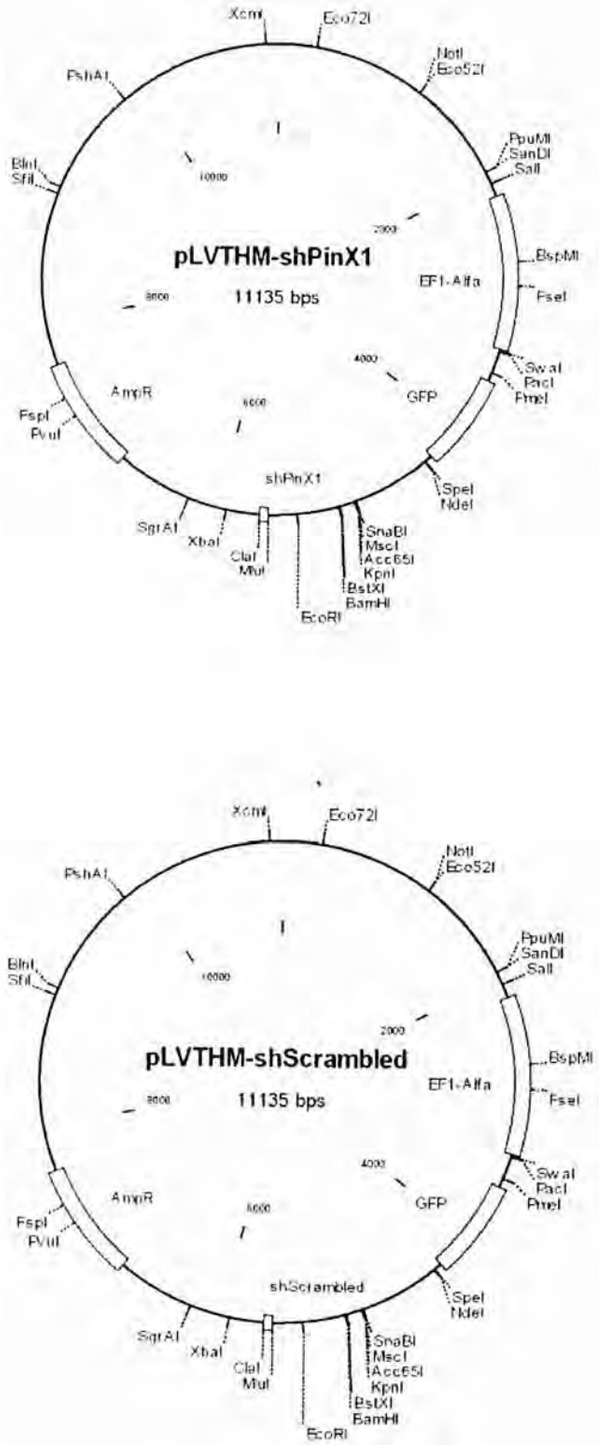
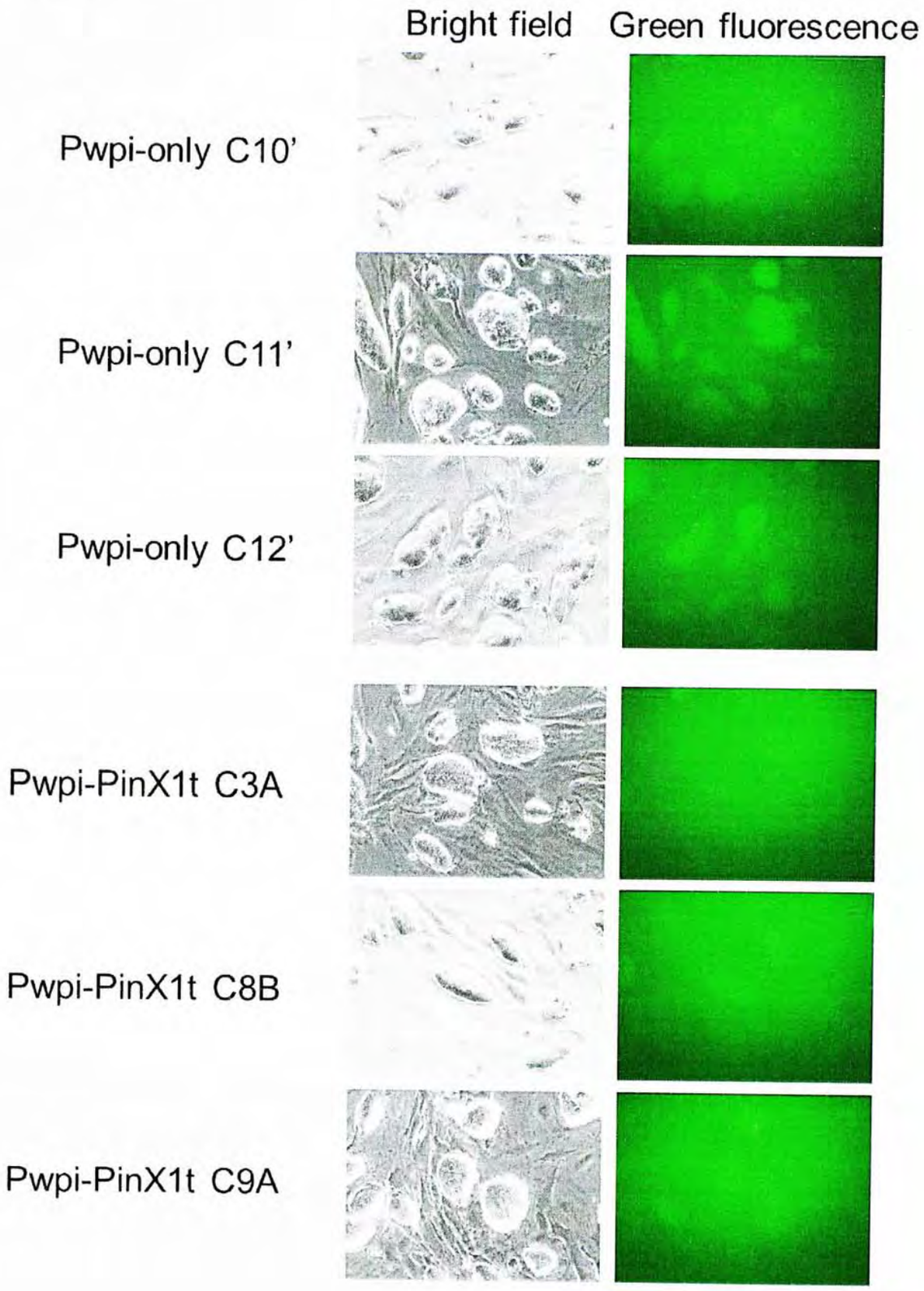


Figure 3.32 Vector maps of (upper panel) pLVTHM-shPinX1 and (lower panel) pLVTHM-shScrambled plasmids.

Figure 3.33


Bright field
Green fluorescence
Pwpi-only C10'
Pwpi-only C11'
Pwpi-only C12'
Pwpi-PinX1t C3A
Pwpi-PinX1t C8B
Pwpi-PinX1t C9A

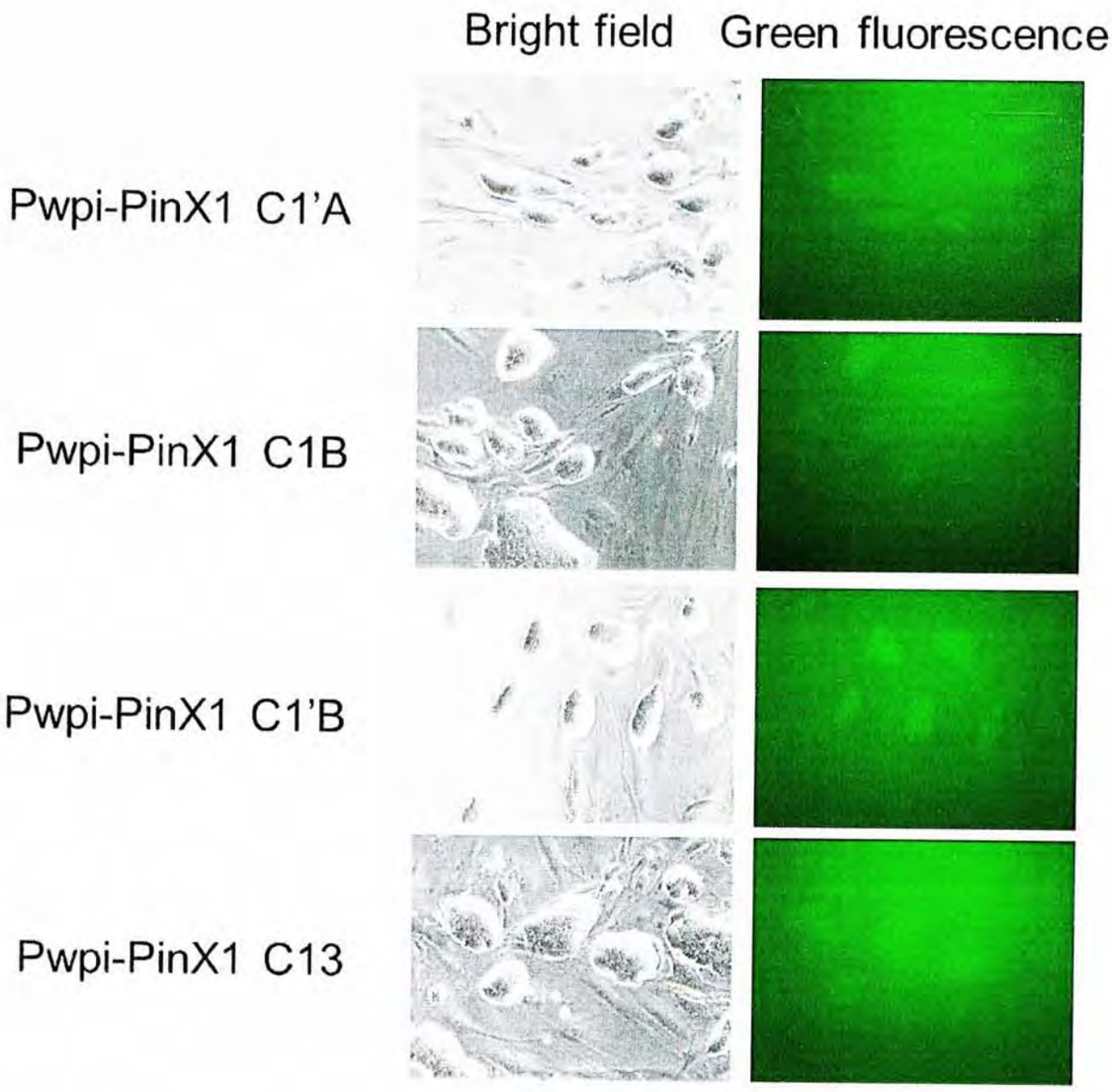


Figure 3.33 Appearance of the established mPinX1, mPinX1t over-expression and control mESC cell lines. 3 stable cell lines were established for control group (Pwpi-only, C10', C11', C12') and mPinX1t over-expression group (Pwpi-PinX1t C3, C7 and C8). 4 stable cells lines were established for mPinX1 over-expression group (Pwpi-PinX1 C1'A, C1B, C1'B and C13).

Figure 3.34

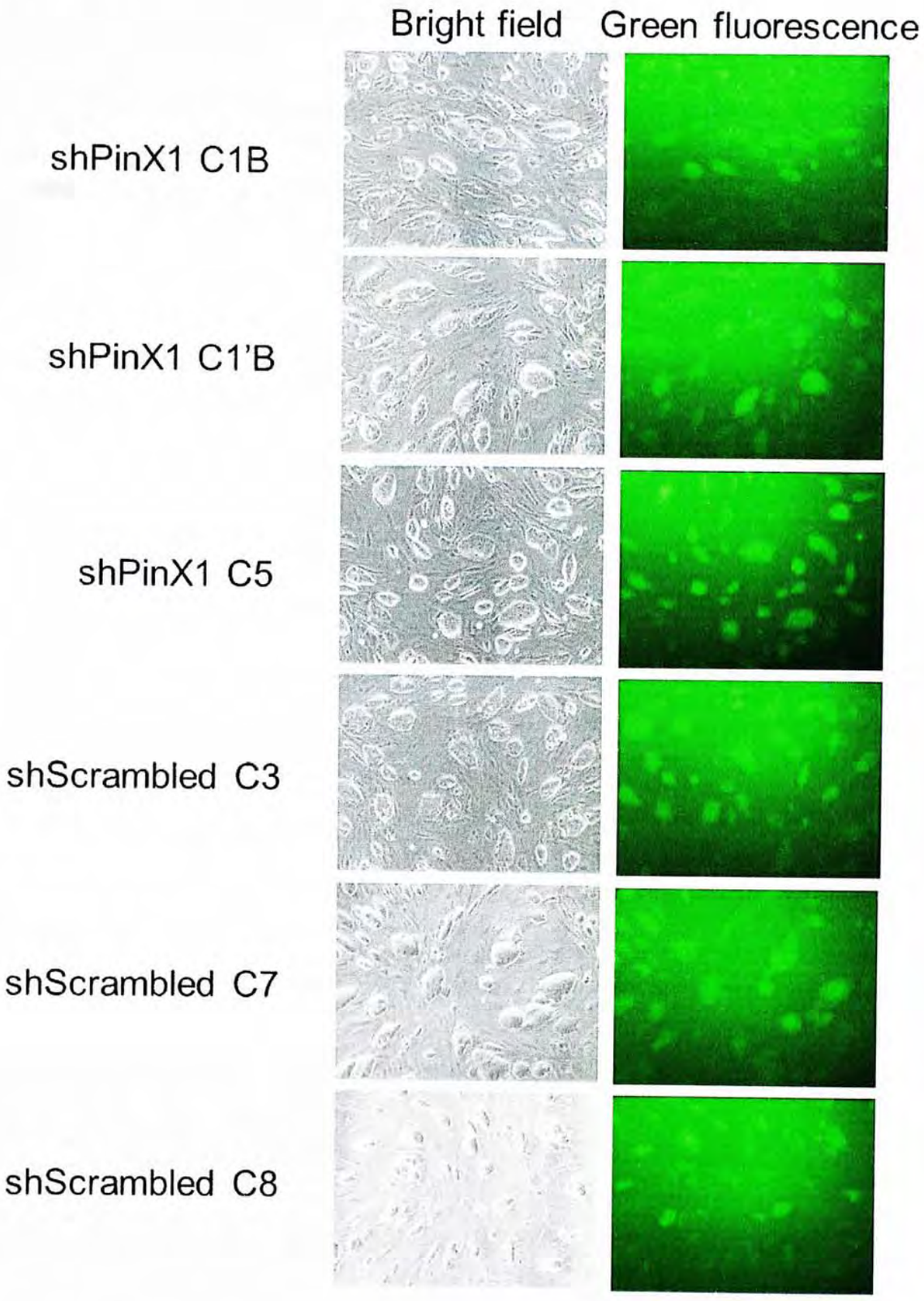


Figure 3.34 Appearance of the established mPinX1 knockdown and control mESC cell lines. 3 stable cells lines were established for mPinX1 knockdown group (shPinX1 C1B, C1'B and C5) and scrambled shRNA control group (shScrambled C3, C7 and C8).

3.9.1 Expression of mPinX1 and mPinX1t at mRNA and protein level in all over-expression stable cell lines

The level of mPinX1 and mPinX1t of the over-expression cell lines at both mRNA and protein levels were examined by RT-qPCR and western blot analysis. For the mPinX1 over-expression cell lines Pwpi-PinX1 C1'A, C13, C1B and C1'B, the mPinX1 mRNA levels were elevated to 19.02-, 23.48-, 20.03- and 18.99-fold, respectively, when compared to that of the average of the control cell lines Pwpi-only C10', C11' and C12' (Fig.3.35). The mPinX1 gene was also up-regulated at protein level for 2.24-, 1.70-, 1.89-, 1.66-fold, respectively, when compared to that of the average of control cell lines, with the appearance of the myc-mPinX1 band on top of the endogenous mPinX1 band (Fig.3.36). Over-expression of mPinX1 in these cell line groups did not affect the expression of mPinX1t at mRNA level. The expression of mPinX1t in these groups were 1.26-, 1.43-, 1.02- and 1.04-fold, respectively, when compared to that of the control groups (Fig.3.35).

For the mPinX1t over-expression cell lines Pwpi-PinX1t C3A, C9A and C8B, the level of mPinX1t gene at mRNA levels were greatly up-regulated for 438.18-, 210.89-, 290.70-fold, respectively, when compared to that of the average of the control cell lines Pwpi-only C10', C11' and C12' (Fig.3.35). The gene was also up-regulated at protein level with the appearance of a band between 20kDa and

30kDa, which is the expected size of the myc-mPinX1t protein and this additional band disappeared when the anti-PinX1 anti-body was pre-incubated with the peptide (Fig.3.37). Its level was increased for 2.68-, 4.55-, 1.57-fold, respectively, when compared to that of the control groups (Fig.3.37). Interestingly, the level of mPinX1 was also up-regulated at mRNA level in these groups for 9.08-, 4.47-, 4.85-fold, respectively, when compared to the average of control groups (Fig.3.35). However, this increase could not be seen at protein level (Fig.3.37).

Figure 3.35

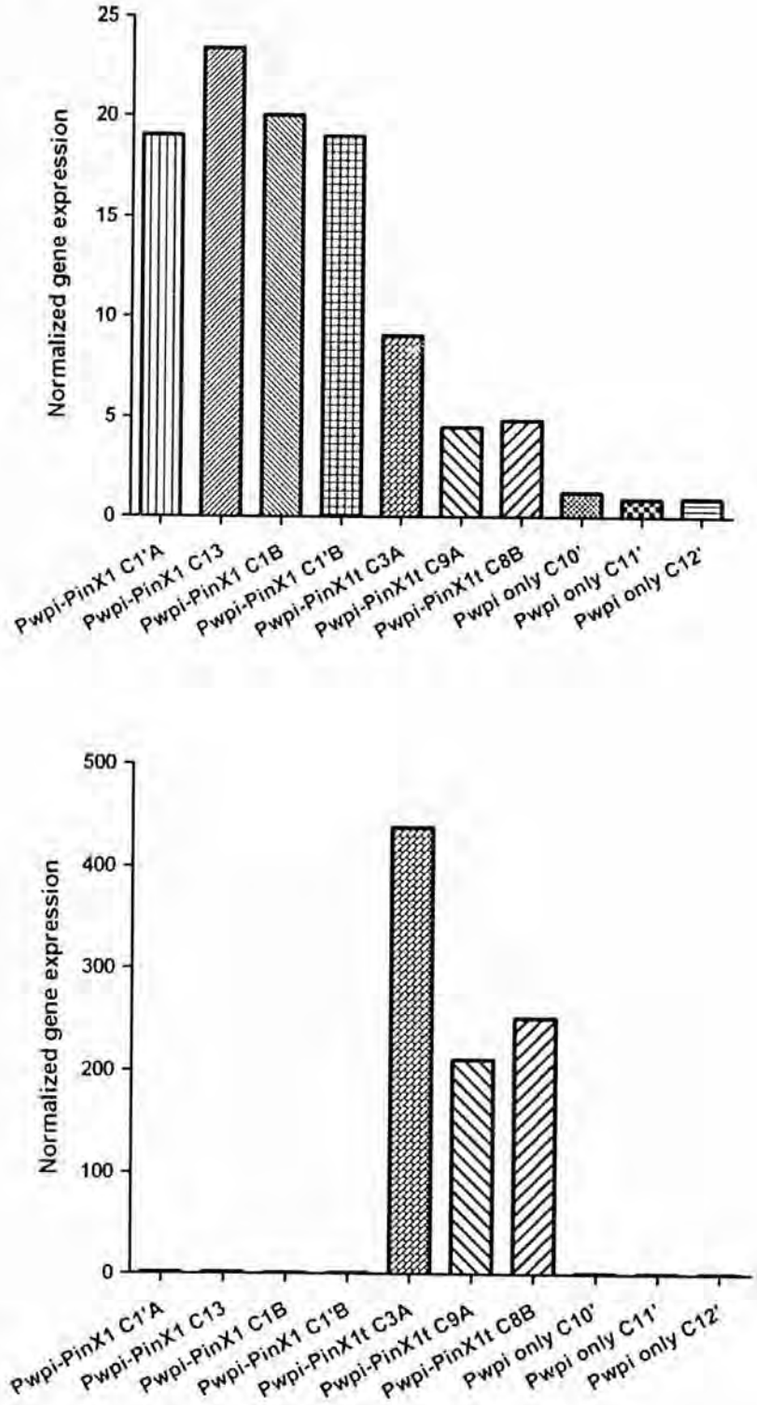


Figure 3.35 RT-qPCR results showing the mPinX1 and mPinX1t mRNA expression level of the stable cell lines. All the gene expression levels were normalized to the average of that of the control (Pwpi only) cell lines. β-Actin was used as a loading control. (Upper panel) mPinX1 expression was increased for ~20 fold in all the mPinX1 over-expression (Pwpi-PinX1) cell lines and increased for ~4-10 fold in all the mPinX1t over-expression (Pwpi-PinX1t) cell lines respectively. (Lower panel) mPinX1t expression was not affected in mPinX1 over-expression cell lines, but increased for ~200-450 fold in the mPinX1t over-expression cell lines.

Figure 3.36

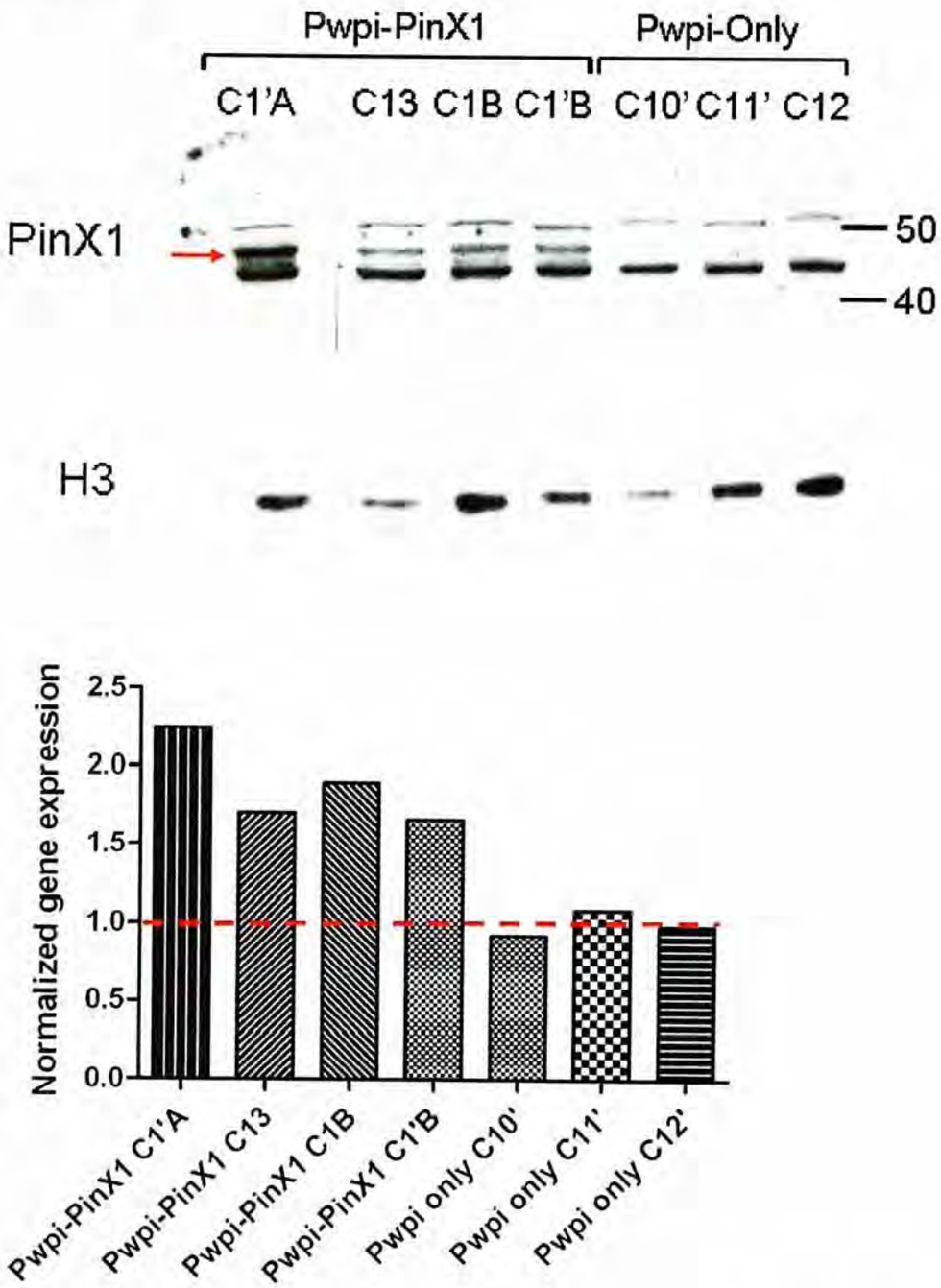


Figure 3.36 Western blot and bar chart showing the mPinX1 protein expression level of the mPinX1 over-expression (Pwpi-PinX1) stable cell lines. All the protein expressions were normalized to the average of the control (Pwpi only) cell lines; H3 was used as a loading control. (Upper panel) An additional myc-mPinX1 band (as indicated by the red arrow) could be seen for all mPinX1 over-expression stable cell lines. (Lower panel) mPinX1 expression was increased for ~1.8-2.5 fold in all the mPinX1 over-expression cell lines.

Figure 3.37

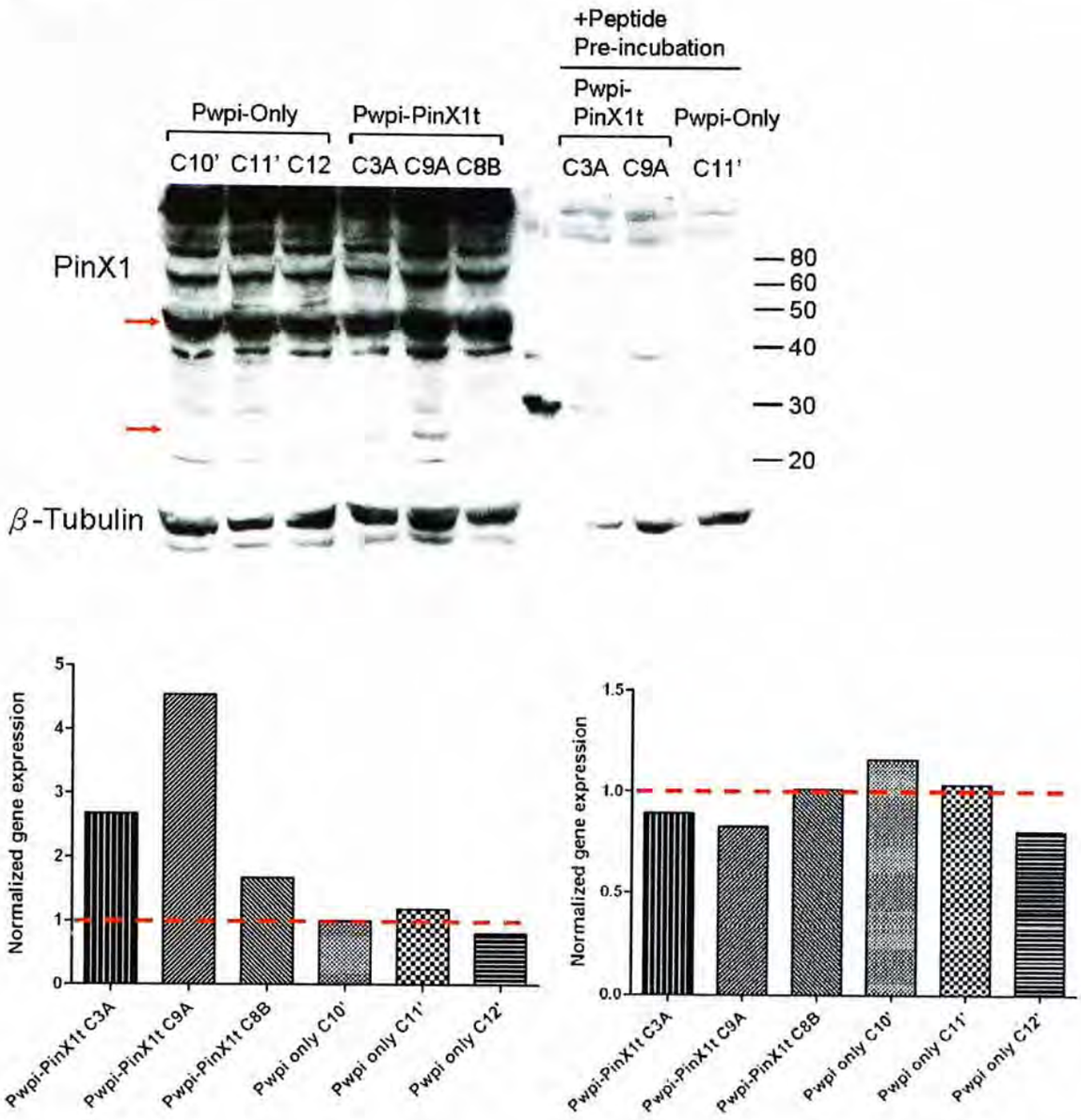


Figure 3.37 Western blot and bar charts showing the mPinX1 and mPinX1t protein expression level of the mPinX1t over-expression (Pwpi-PinX1t) stable cell lines. All the protein expressions were normalized to the average of that of the control (Pwpi-only) cell lines; β-Tubulin was used as a loading control. (Upper panel) An additional myc-mPinX1t band (as indicated by the lower red arrow) could be seen for all mPinX1t over-expression stable cell lines. The additional band disappeared when the anti-PinX1 antibody was pre-incubated with peptide. On the other hand, there is no observable change in the expression of mPinX1 (as indicated by the upper red arrow). (Lower left panel) mPinX1t expression was increased for ~1.5-4.6 fold in all the mPinX1t over-expression cell lines. (Lower right panel) There was no significant change in the mPinX1 expression level in the mPinX1t over-expression cell lines.

3.9.2 Expression of mPinX1 and mPinX1t at mRNA and protein level in mPinX1 knockdown stable cell lines

The expression of both mPinX1 and mPinX1t gene were examined in the mPinX1 stable knockdown cell lines. For mPinX1, the gene was successfully knocked down at both mRNA and protein levels. The mPinX1 mRNA level for shPinX1 C1B, C1'B and C5 was diminished to 0.63-, 0.17- and 0.16- fold respectively, when compared to that of the average of the control cell lines shScrambled C3, C7 and C8 (Fig.3.38). At protein level, the expression of mPinX1 was also diminished to 0.51-, 0.48- and 0.49-fold when compared to the average of that of the control cell lines (Fig.3.39). For mPinX1t gene, it also showed a slight knockdown in the shPinX1 cell lines to 0.81-, 0.65- and 0.63- fold respectively when compared to control cell lines. (Fig.3.38). The degree of knockdown of mPinX1t was much lower than that of mPinX1.

Figure 3.38

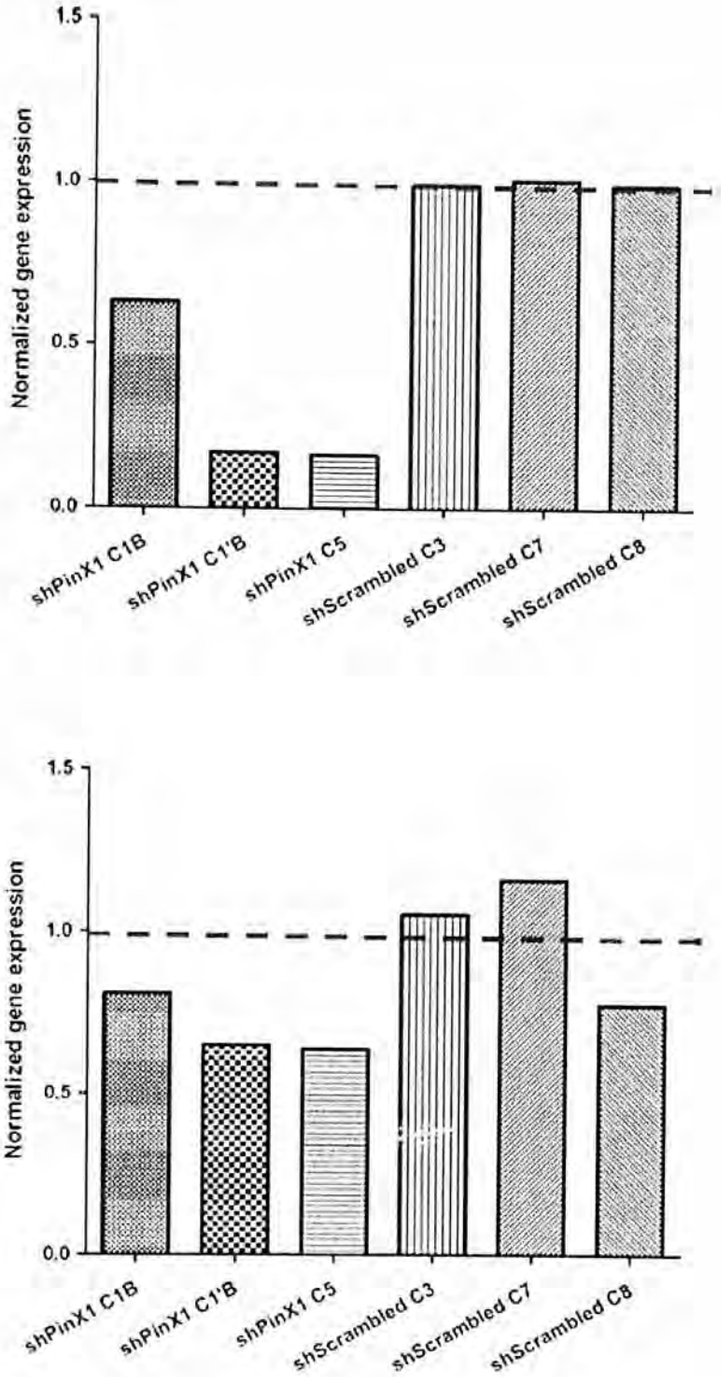


Figure 3.38 RT-qPCR result showing the mPinX1 and mPinX1t mRNA expression level of the mPinX1 knockdown stable cell lines. All the gene expression levels were normalized to the average of that of the control (shScrambled) cell lines. β-Actin was used as a loading control. (Upper panel) mPinX1 expression was down-regulated to ~0.1-0.6 fold in all the mPinX1 knockdown (shPinX1) cell lines. (Lower panel) mPinX1t expression had a minor effect of down-regulation to ~0.6-0.8 fold in all the mPinX1 knockdown cell lines.

Figure 3.39

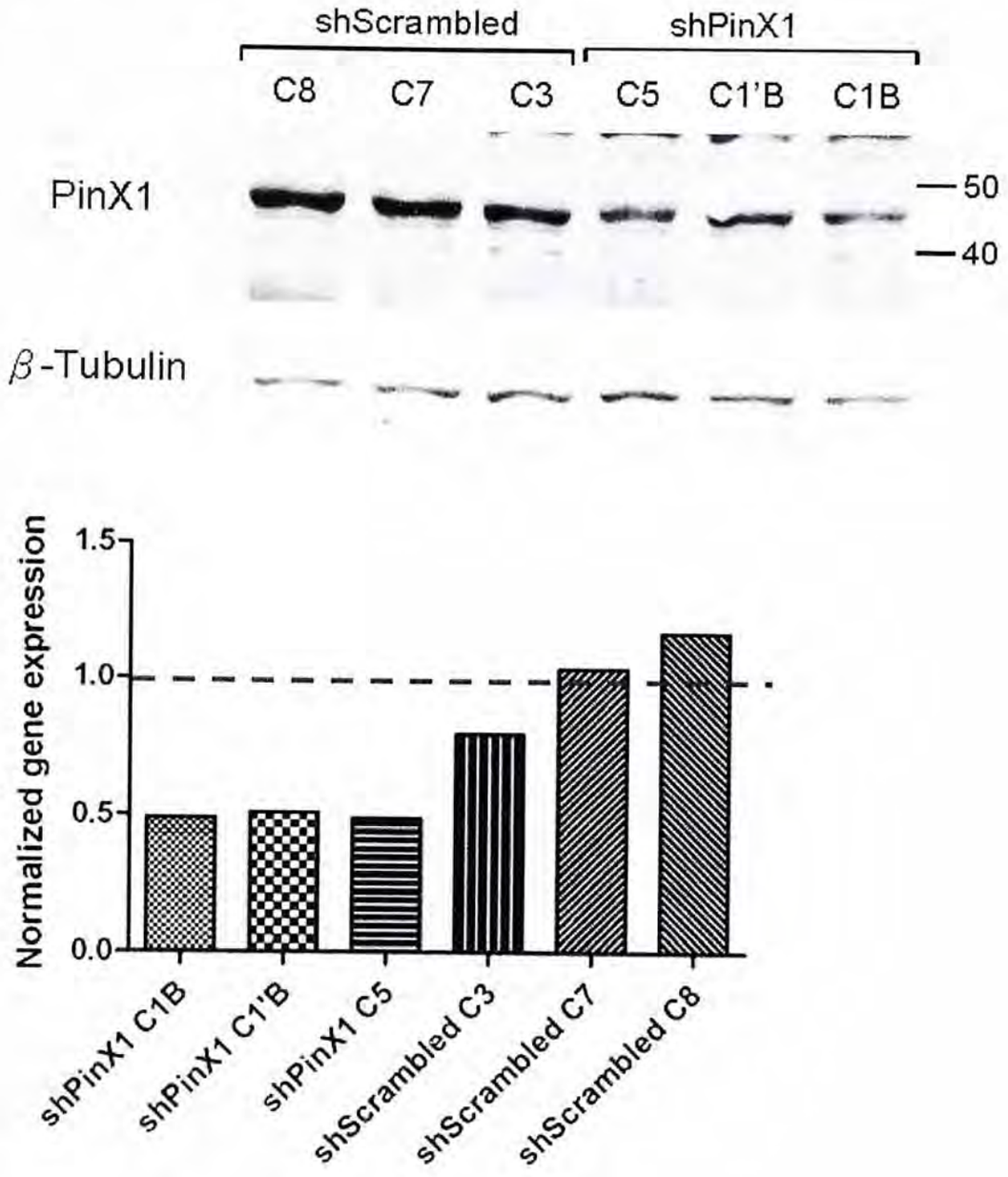


Figure 3.39 Western blot and bar chart showing the mPinX1 protein expression level of the mPinX1 knockdown (shPinX1) stable cell lines. All the protein expressions were normalized to the average of the control (shScrambled) cell lines; β-Tubulin was used as a loading control. mPinX1 expression was decreased to ~0.5 fold in all the mPinX1 knockdown cell lines.

3.9.3 Proliferation of all stable cell lines

To study the long term effect on cell proliferation, the stable cell lines were kept on being passaged for 38-39 passages starting from the day of their establishment. The number of cells was counted by trypan blue exclusion assays every time when the cells were passaged. At passages ~10, ~20 and ~30, MTT assay was also done to examine the proliferation rate of the cells.

For the stable cell lines, both over-expression of mPinX1 or mPinX1t, and the knock-down of mPinX1 did not cause a significant change in cell proliferation in the long run as determined by trypan blue exclusion assays (Fig.3.40 & Fig.3.41). The MTT assay results also showed that the proliferation rate of the stable over-expression/ knockdown cell lines were similar to that of the control cell lines at all the passages examined (n=1-2, Fig.3.42 and Fig.3.43). All these results showed that, unlike transient over-expression or knockdown, stable over-expression or knockdown of the genes did not cause any significant change in cell proliferation in the long term.

Figure 3.40

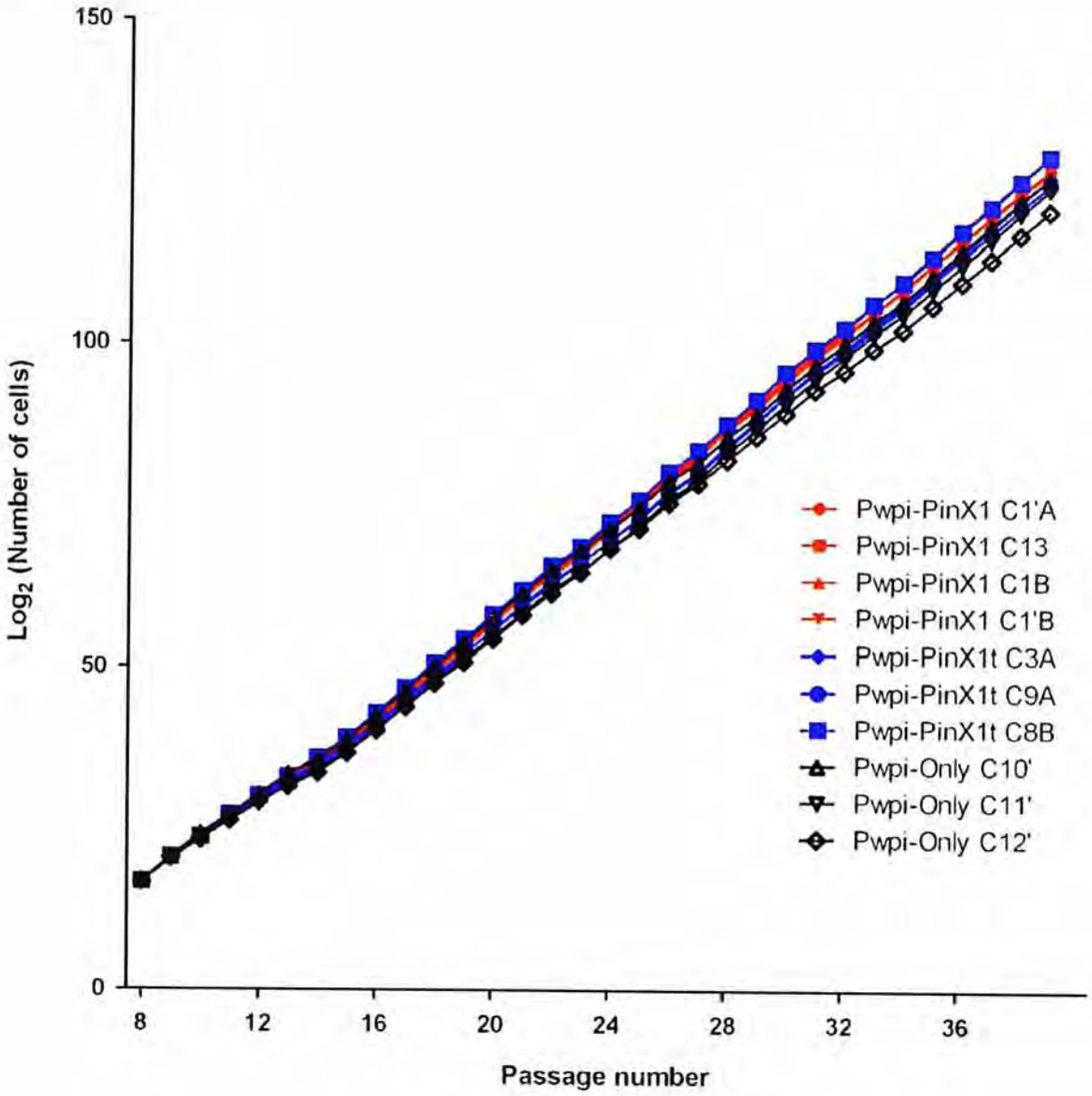


Figure 3.40 Proliferation curve of 39 passages of mPinX1 over-expression (Pwpi-PinX1), mPinX1t over-expression (Pwpi-PinX1t) and control (Pwpi only) cell lines. The graph was plotted with Log_2 (total number of cells) against the passage number. There was no significant difference in the rate of proliferation between the cell lines within the 39 passages.

Figure 3.41

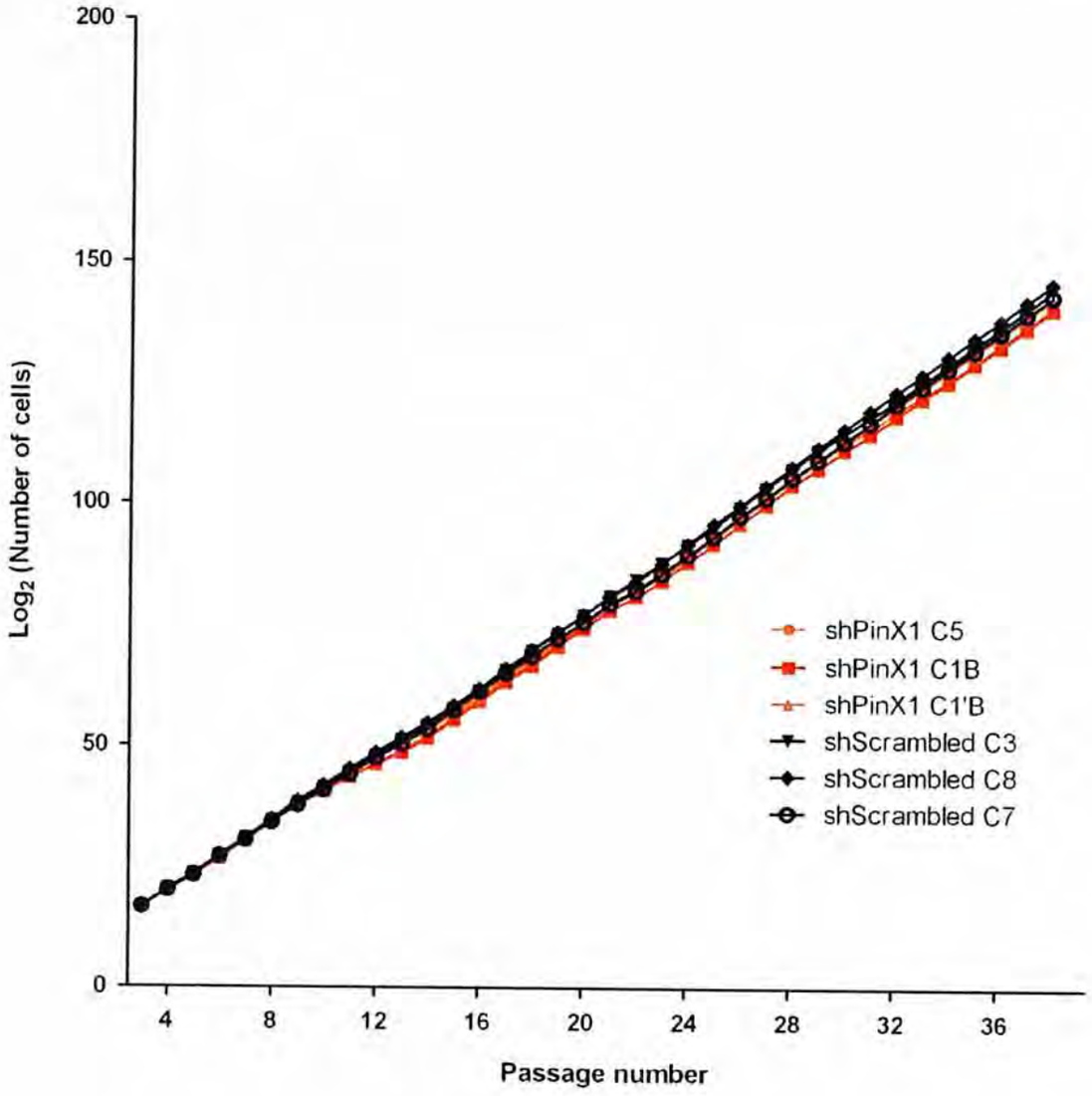


Figure 3.41 Proliferation curve of 38 passages of mPinX1 knockdown (shPinX1) and control (shScrambled) cell lines. The graph was plotted with Log_2 (Total number of cells) against the passage number. There was no significant difference in the rate of proliferation between the cell lines within the 38 passages.

Figure 3.42

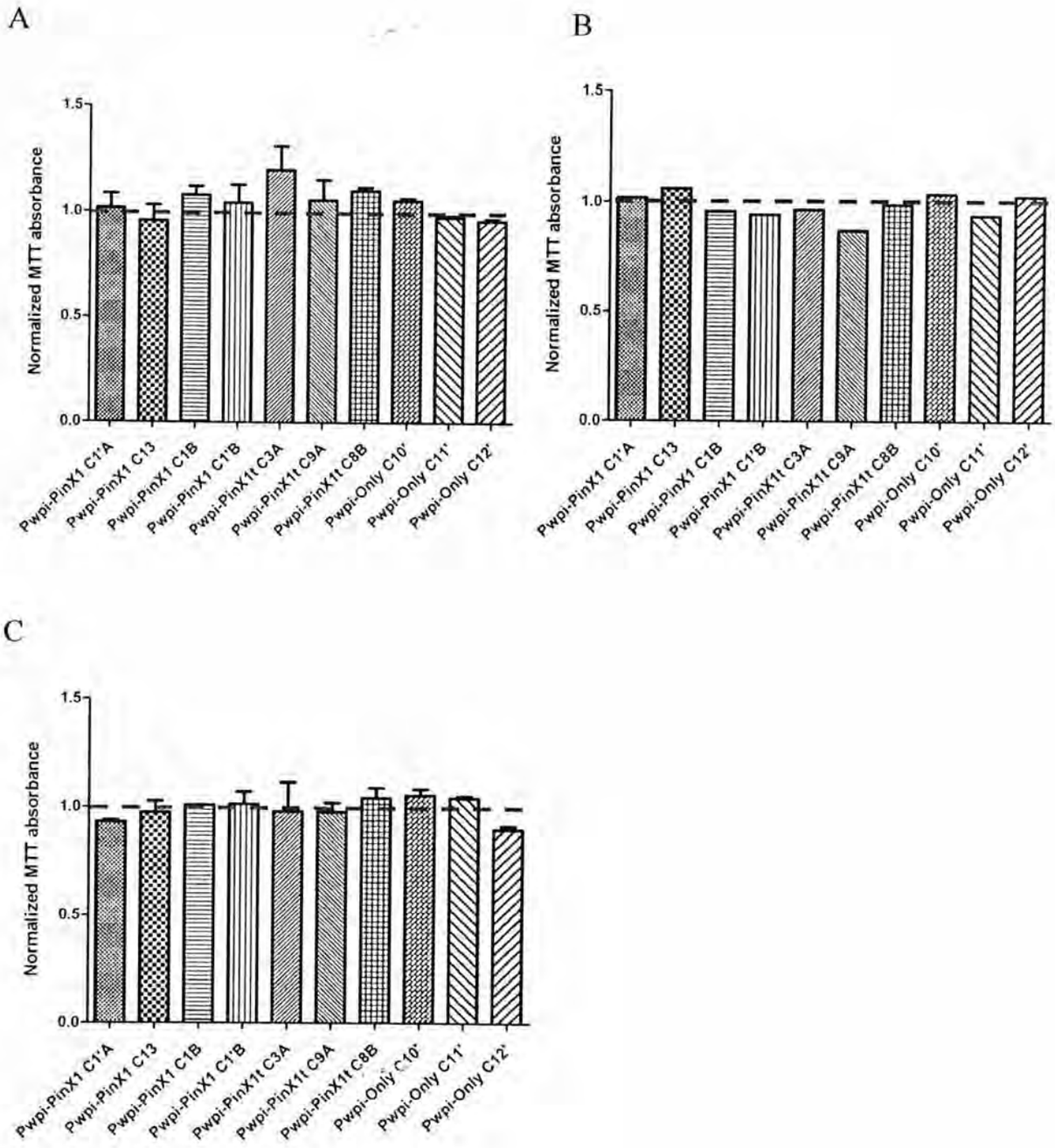


Figure 3.42 MTT result of mPinX1 over-expression (Pwpi-PinX1), mPinX1t over-expression (Pwpi-PinX1t) and control (Pwpi-only) cell lines at (A) passages ~10, (B) ~20 and (C) ~30. The MTT absorbance was normalized to the average of that of the control cell lines. There was no significant difference in the MTT absorbance between the cell lines in all these passages. For passage ~10, values are mean ± S.E.M. of 3 experiments; for passage ~20, values are from 1 experiment; for passage ~30, mean ± S.E.M. of 2 experiments.

Figure 3.43

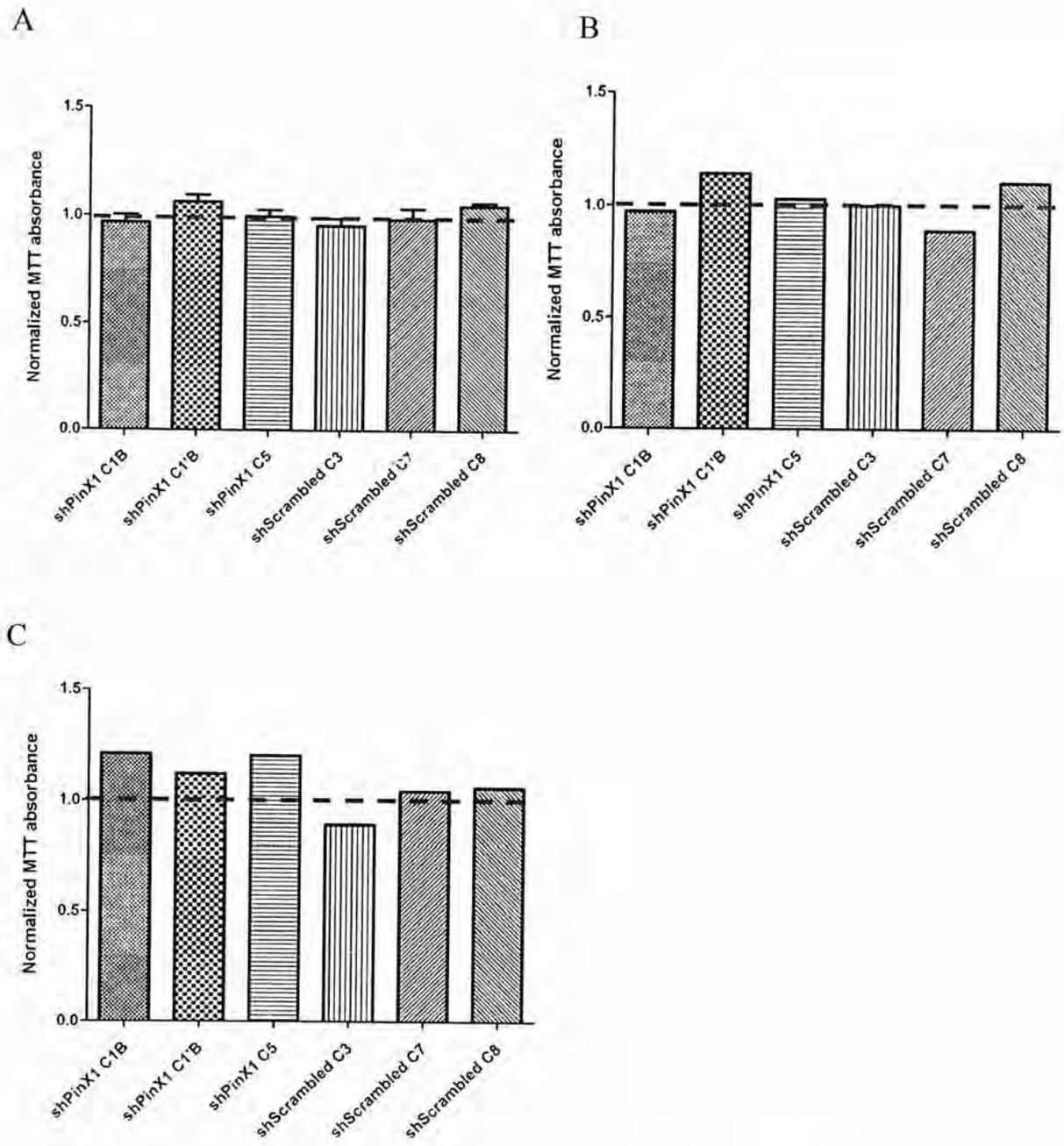


Figure 3.43 MTT result of mPinX1 knockdown (shPinX1) and control (shScrambed) cell lines at (A) passages ~10, (B) ~20 and (C) ~30. The MTT absorbance was normalized to the average of control cell lines. There was no significant difference in the rate of proliferation between the cell lines in all these passages. For passage ~10, values are mean ± S.E.M. of 2 experiments; for passage ~20 and passage ~30, values are from 1 experiment.

3.9.4 Telomerease activity of all stable cell lines

TRAP assay was done to examine the telomerase activity of the stable cell lines. The results showed that for both the over-expression and the knock-down cell lines, there was no significant change of the telomerase activity (n=1-4, Fig.3.44). This is different from the transient over-expression and knock-down results, in which the telomerase activity was affected by the mPinX1 expression.

Figure 3.44

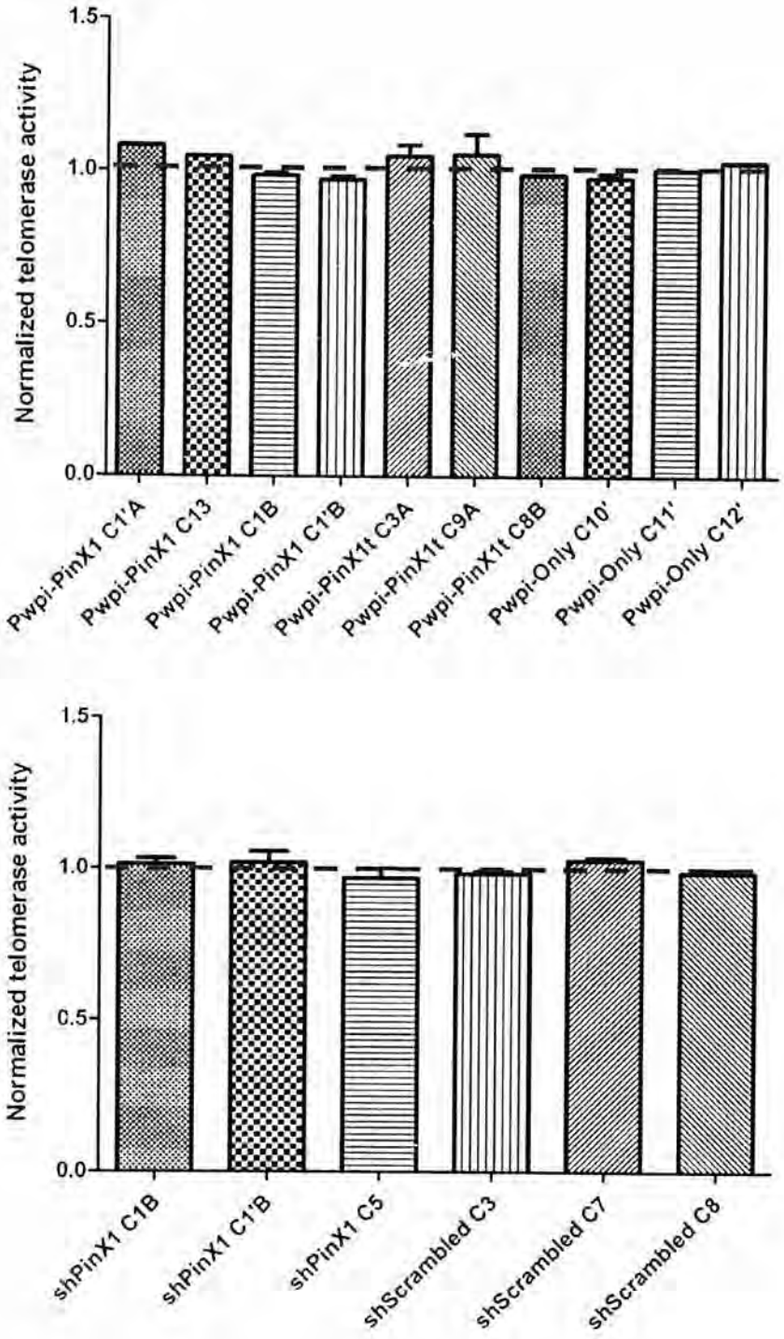


Figure 3.44 TRAP assay result of all the stable cell lines. For mPinX1 over-expression (Pwpi-PinX1), mPinX1t over-expression (Pwpi-PinX1t) stable cell lines, the telomerase activity was normalized to the average of control (Pwpi-only) cell lines. (Upper panel) For mPinX1 knockdown (shPinX1) cell lines, the telomerase activity were normalized to the average of control (shScrambled) cell lines. (Lower panel) There was no significant difference in the telomerase activity of all the stable cell lines. For over-expression groups, the values mean ± S.E.M. of 1-2 experiments. For knockdown groups, the values mean ± S.E.M. of 4 experiments.

3.9.5 Cell cycle distribution of all stable cell lines

Flow cytometry was done to study the cell cycle distribution of the stable cell lines. All the groups of cells were collected at P29 for PI staining followed by flow cytometry. The results showed that the cell cycle distribution of all the cell lines were similar, with ~60% of cells in S phase and ~20% of cells in G_0/G_1 phase and S phase (n=1, Fig.3.45, Fig.3.46 & Fig.3.47). Since only one trial was done, more independent experiments are needed to confirm the results in the future.

Figure 3.45

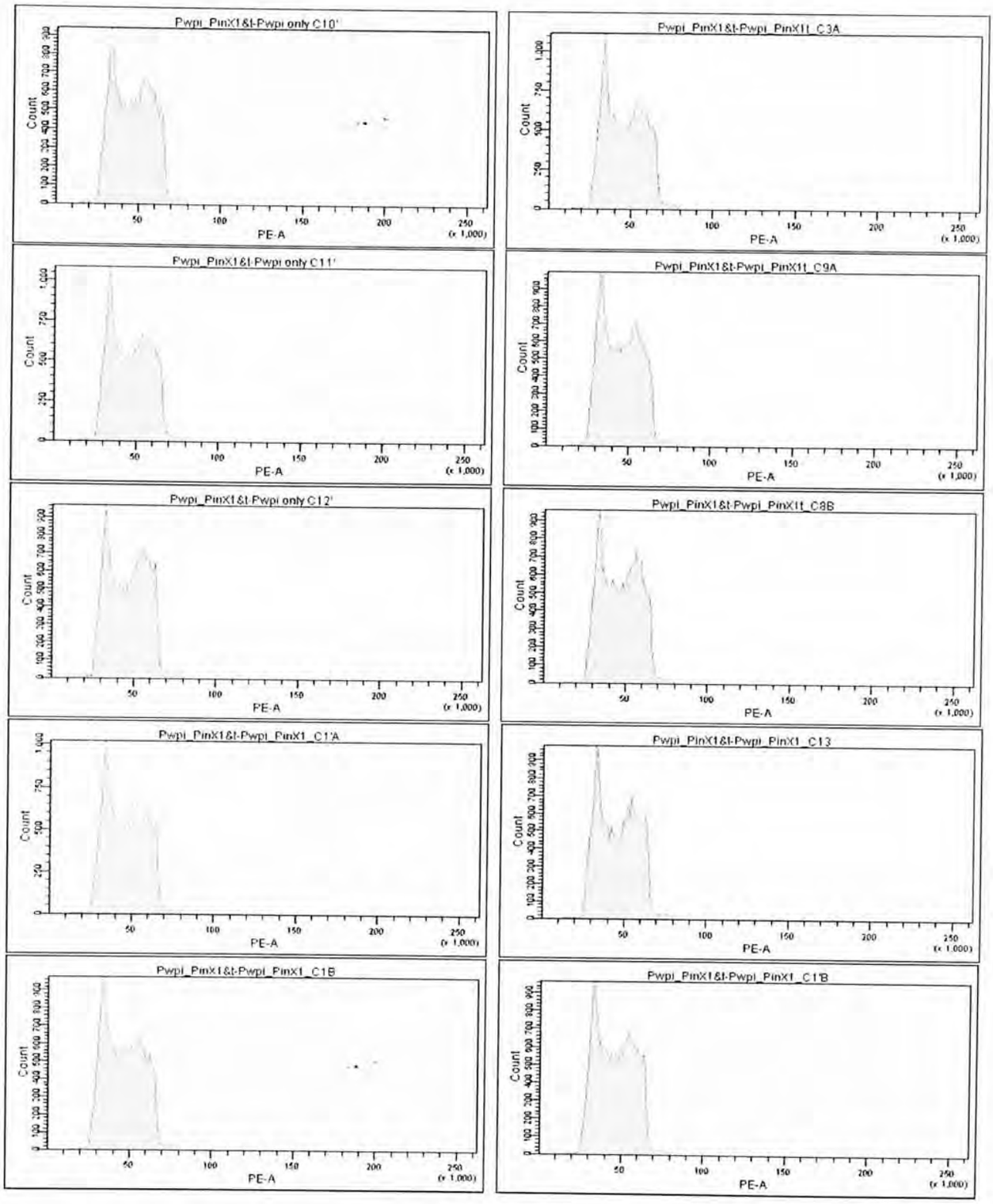


Figure 3.45 The cell cycle profiles of the mPinX1 over-expression (Pwpi-PinX1), mPinX1t over-expression (Pwpi-PinX1t) and control (Pwpi-only) cell lines.

Figure 3.46

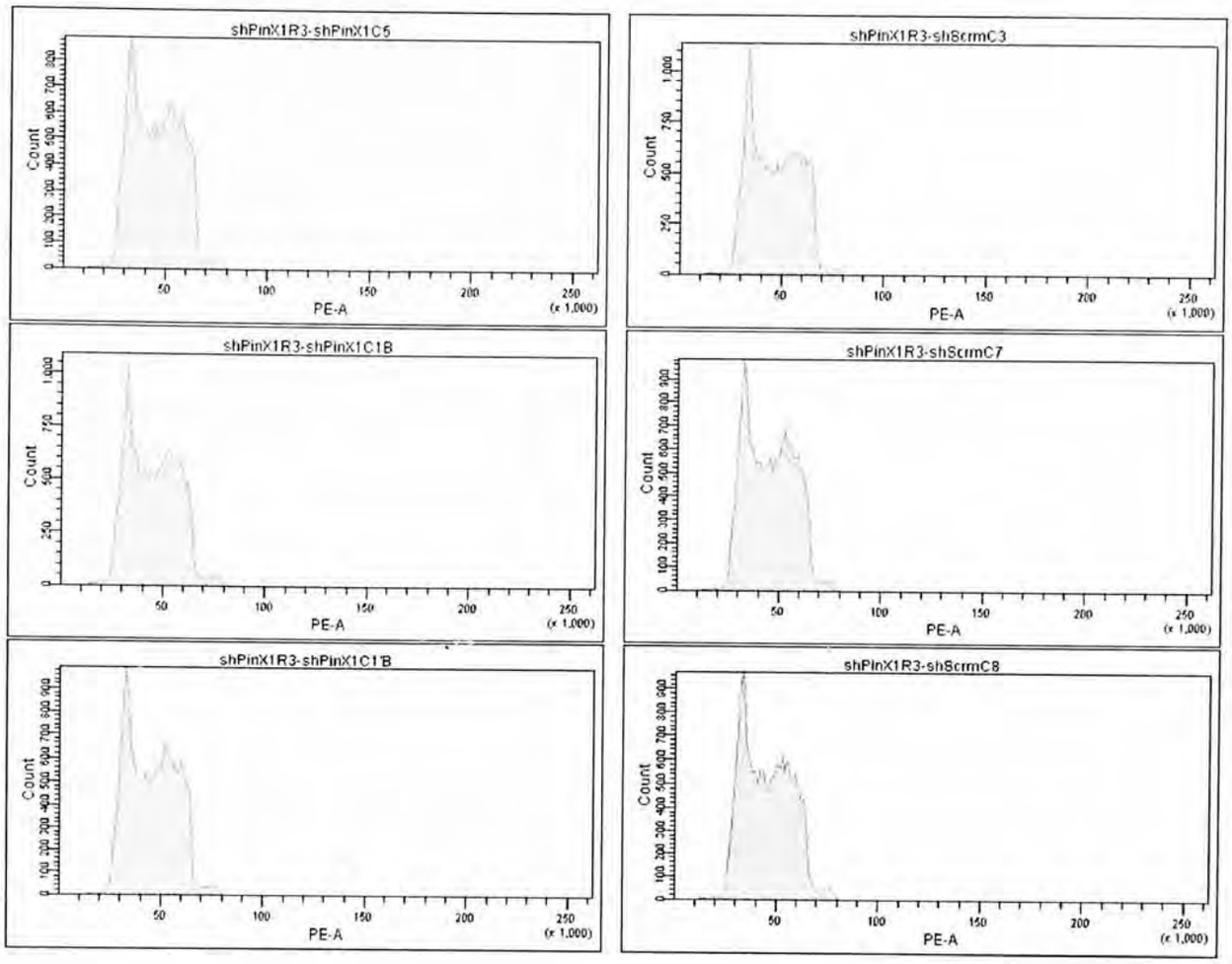


Figure 3.46 The cell cycle profiles of the mPinX1 knockdown (shPinX1), and control (shScrambled) cell lines.

Figure 3.47

Percentage (%)	**Pwpi-mPinX1**				**Pwpi-mPinX1t**			**Pwpi-only**		
	C1'A	**C13**	**C1B**	**C1'B**	**C3A**	**C9A**	**C8B**	**C10'**	**C11'**	**C12'**
G0/G1	16.80	18.07	17.97	17.37	19.28	17.91	16.42	16.48	18.33	16.68
S	65.58	61.14	67.24	64.96	62.46	63.63	63.21	64.22	63.32	60.30
G2/M	17.62	20.78	14.79	17.67	18.26	18.45	20.37	19.31	18.36	23.02

Percentage (%)	**shPinX1**			**shScrambled**		
	C1B	**C1'B**	**C5**	**C3**	**C7**	**C8**
G0/G1	17.99	19.29	18.20	19.50	19.92	18.64
S	63.32	62.34	63.20	62.94	64.53	62.77
G2/M	18.69	18.37	18.60	17.55	15.55	18.59

Figure 3.47 (Upper panel) The percentage of the G_0/G_1, S, and G_2/M phase populations of mPinX1 over-expression (Pwpi-PinX1), mPinX1t over-expression (Pwpi-PinX1t) and its control (Pwpi-only) cell line. (Lower panel) The percentage of the G_0/G_1, S, and G_2/M phase populations of mPinX1 knockdown (shPinX1) and its control (shScrambled) cell lines. There was no significant change of cell cycle distribution between all groups.

3.9.6 Pluripotency of all stable cell lines

Western blot was done for the stable cell lines on pluripotent markers to see if there was a change in pluripotency upon altering the expression of the mPinX1 and mPinX1t. Three pluripotent markers Oct-4, Klf-4, and Sox-2 were chosen. β-Tubulin were used as the loading control. Preliminary data showed that there was no obvious and consistent change in the expression of pluripotent markers in different groups. The inconsistent change in the expressions of pluripotent markers in different clones of the same group might be caused by the effect of random insertion of the exogenous gene in the genome. Since only one trial was done, in the future, more independent experiments are needed to confirm the results (Fig.3.48 & Fig.3.49).

Figure 3.48

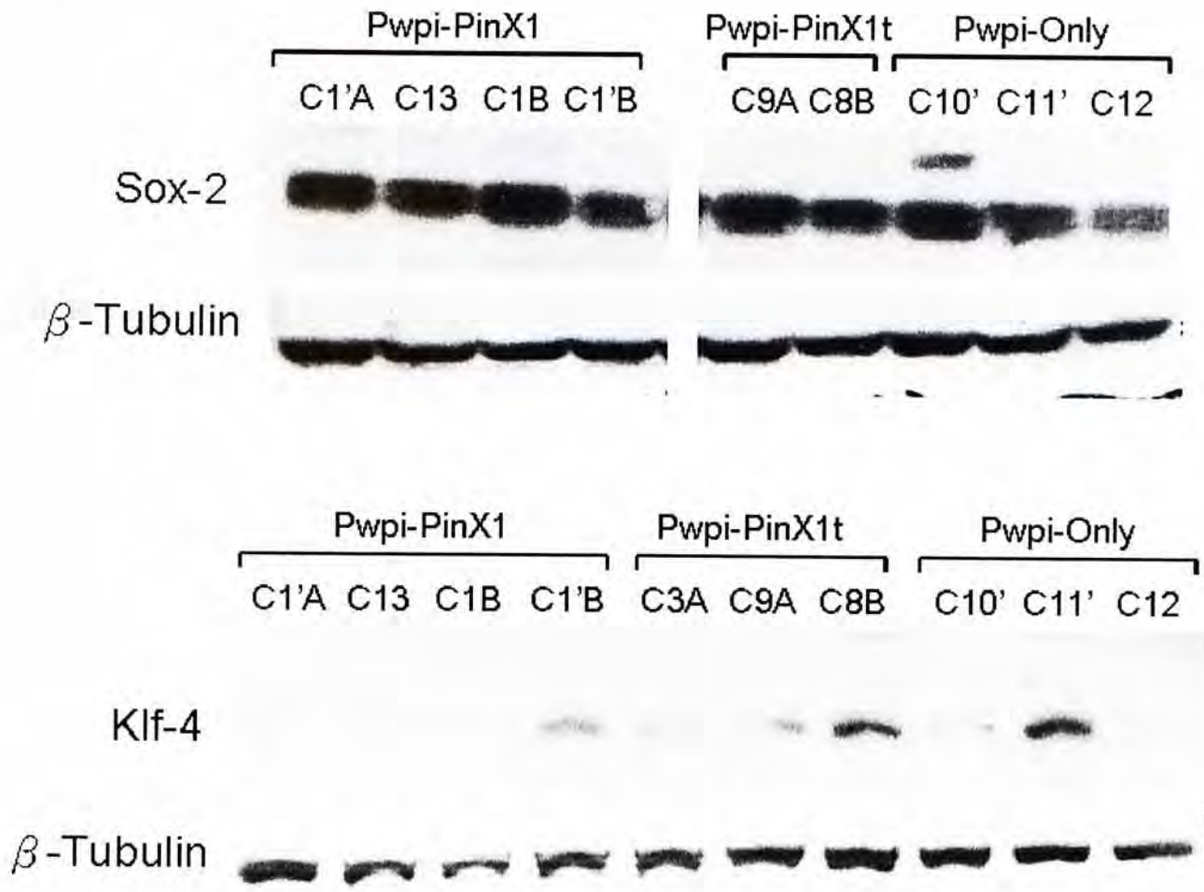


Figure 3.48 Western blots showing the expression of pluripotent markers Sox-2 and Klf-4 in mPinX1 over-expressed (Pwpi-PinX1), mPinX1t over-expressed (Pwpi-PinX1t) and the control (Pwpi only) cell lines. β-Tubulin was used as the loading control.

Figure 3.49

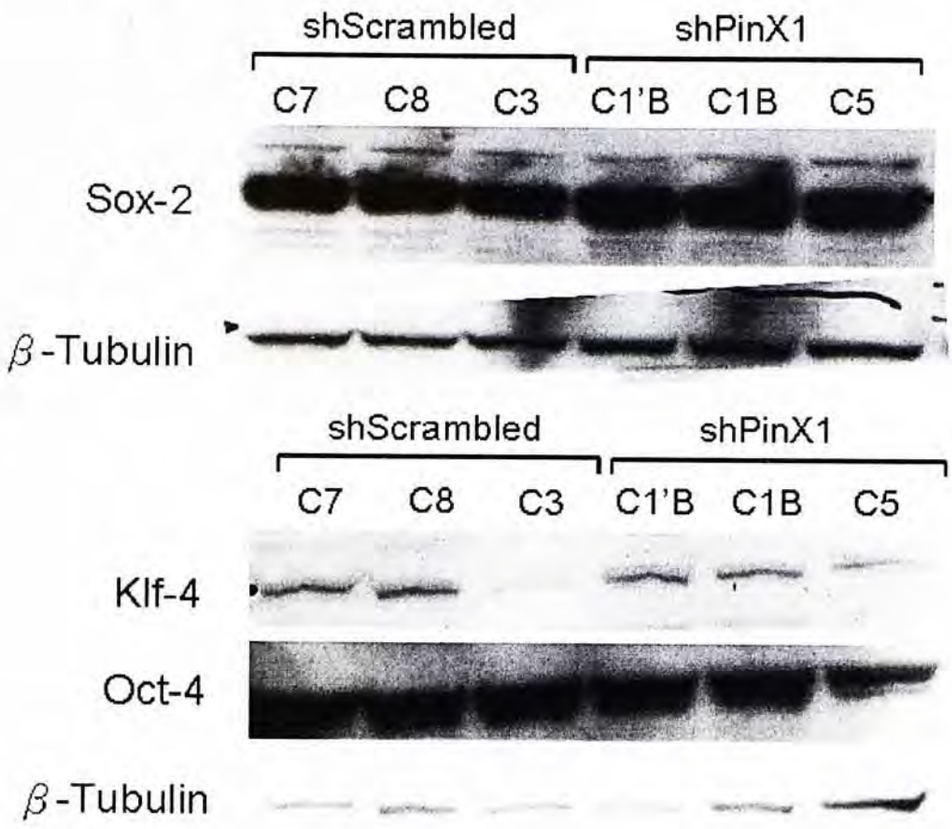


Figure 3.49 Western blot showing the expression of pluripotent markers Sox-2, Klf-4 and Oct-4 in mPinX1 knockdown (shPinX1) and the control (shScrambled) cell lines. β-Tubulin was used as the loading control.

3.9.7 Differentiation of the stable cell lines

To study the role of mPinX1 and mPinX1t in differentiation process, two clones from each group of stable cell line were chosen for differentiation by hanging-drop method. The clones chosen were C11' and C12' for Pwpi-only, C1'A, C13 for Pwpi-PinX1, C3A and C9A for Pwpi-PinX1t, C1'B and C5 for shPinX1 and C7 and C8 for shScrambled)

3.9.7.1 Size of EBs formed from stable cell lines at Day 7

At Day 7 of differentiation, the size of EBs formed was measured. For each group, around 40-60 EBs were measured from each stable cell line. Both over-expression of mPinX1 or mPinX1t, and the knock-down of mPinX1 did not cause a significant change in the size of EBs formed, they all had a diameter of around 300μm (n=2, Fig.3.50 & Fig.3.51). All the EBs still got green fluorescent signals, meaning that the exogenous mPinX1 gene, mPinX1t gene and shPinX1 were still robustly expressed after differentiation (Fig.3.50).

3.9.7.2 Beating curves of the stable cell lines derived EBs

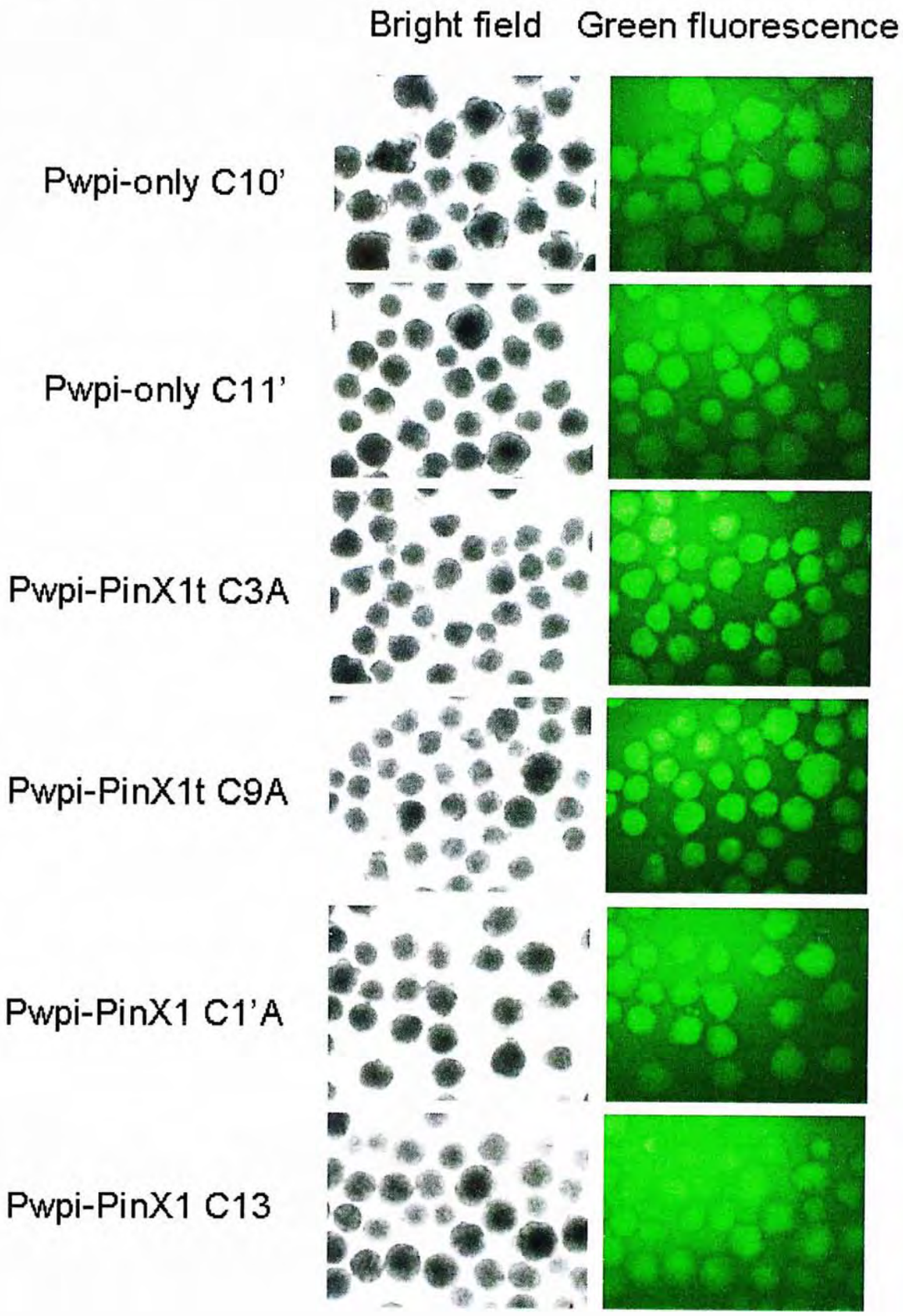
At Day 7 of differentiation, 24 EBs from each group were attached onto 24-well plates. From Day 7+1 to Day 7+12, the EBs were examined under microscope

everyday to count the number of beating EBs.

For the over-expression groups, Pwpi-only control group and mPinX1 over-expression group started to beat at Day 7+4, while for Pwpi-mPinX1t showed a trend of delayed onset of beating, which started at Day 7+5. For Pwpi-only control group and mPinX1t over-expression group, all clones reached maximum beating at around Day 7+10. For mPinX1 over-expression group, both clones reached 100% beating at Day 7+7, showing a trend of earlier time of reaching of maximum beating. All groups reached percentage of beating EBs of above 95% (n=2, Fig.3.52).

For the knock-down groups, all the EBs started to beat at around Day 7+4 and reached a maximum beating at around Day 7+10. For the two clones in shScrambled group, C7 reached a maximum of 98% beating, while C8 reached a maximum of 100% beating. For the two clones in shPinX1 group, C1'B reached a maximum of 77% beating, while C8 reached a maximum of 87% beating. Knockdown of mPinX1 showed a trend of decreased maximum percentage of beating EBs formed (n=2, Fig.3.53).

Figure 3.50


Bright field
Green fluorescence
Pwpi-only C10'
Pwpi-only C11'
Pwpi-PinX1t C3A
Pwpi-PinX1t C9A
Pwpi-PinX1 C1'A
Pwpi-PinX1 C13

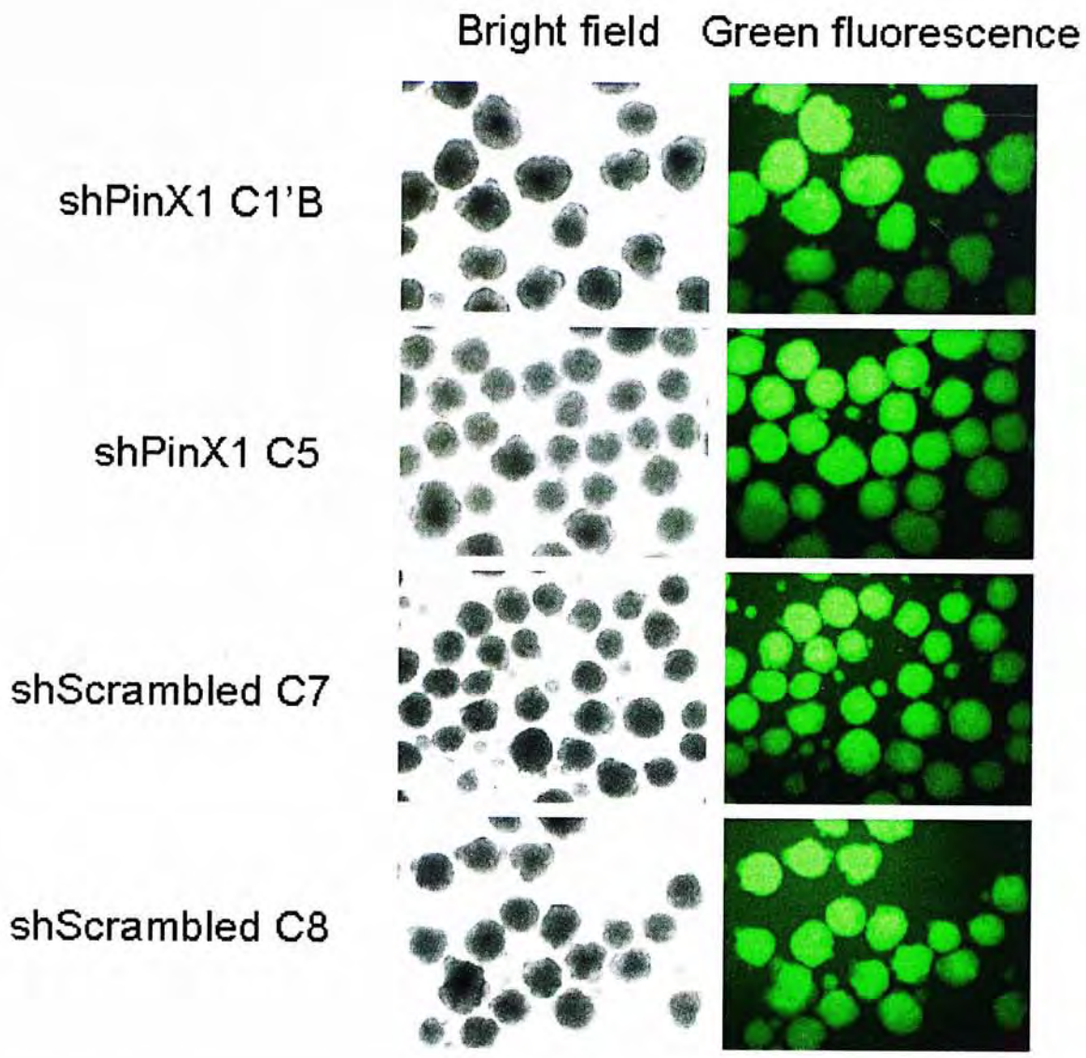


Figure 3.50 Representative photos showing the size of EBs from each cell line at Day 7 after differentiation at bright field and green florescence view.

Figure 3.51

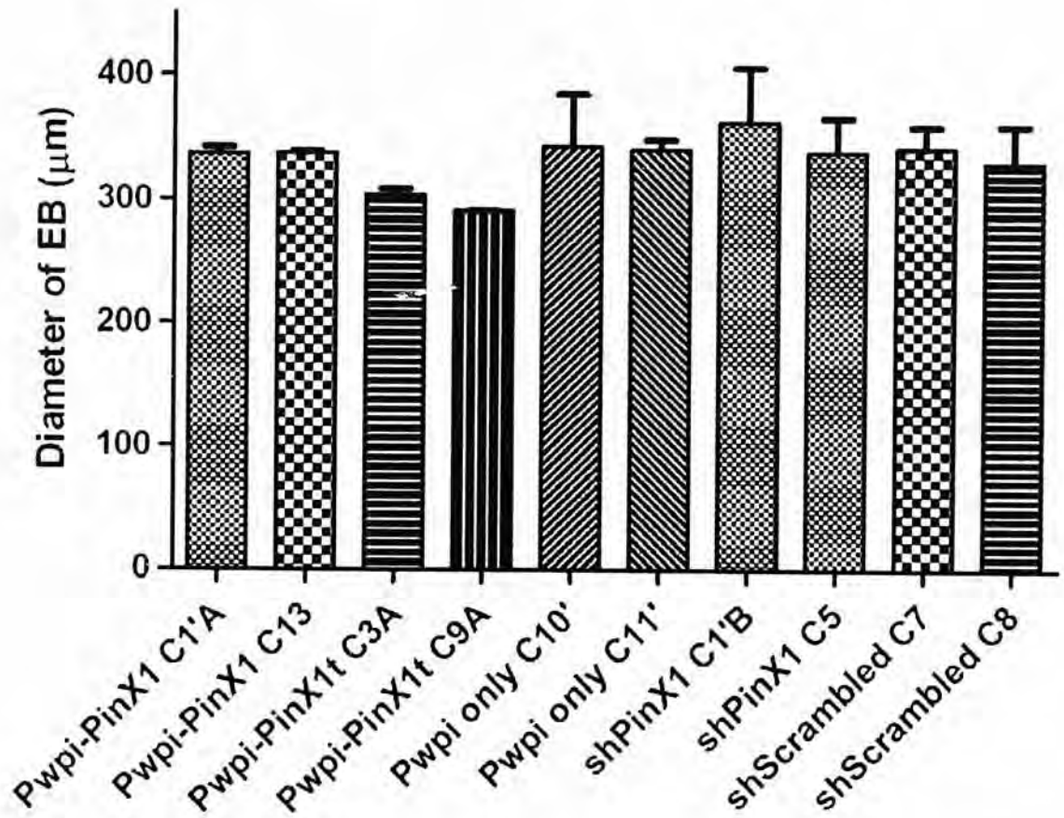


Figure 3.51 Bar chart showing the size of EBs from each cell line at Day 7 after differentiation. The sizes of EBs of all groups were around 300μm. Values are mean ± S.E.M. of 2 experiments.

Figure 3.52

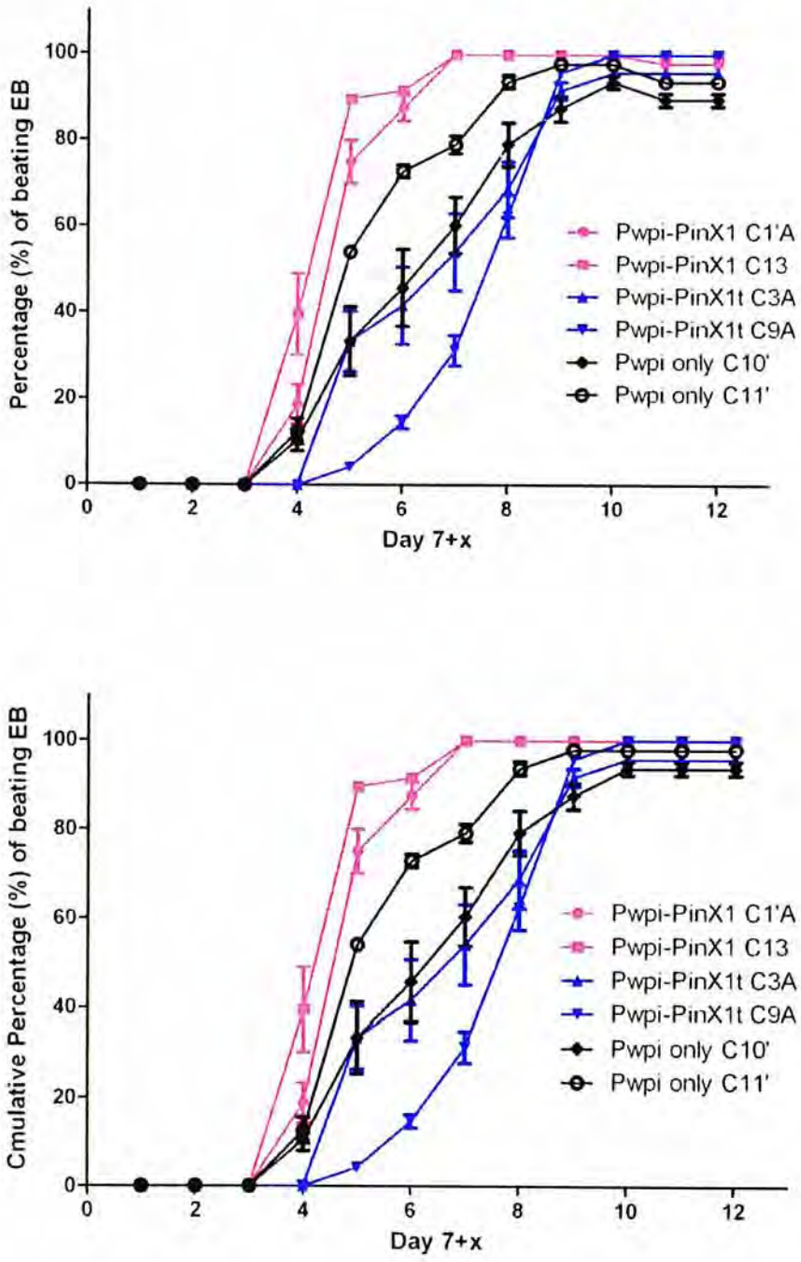


Figure 3.52 (Upper panel) Beating curve and (lower panel) cumulative beating curve of mPinX1 over-expression (Pwpi-PinX1), mPinX1t over-expression (Pwpi-PinX1t) and control (Pwpi only) cell lines. Pwpi-only control group and mPinX1 over-expression group started to beat at Day 7+4, while for Pwpi-mPinX1t the beating started at Day 7+5. For control group and mPinX1t over-expression group, all clones reached a maximum beating at around Day 7+10. For mPinX1 over-expression group, both clones reached 100% beating at Day 7+7, which was earlier than that of control. All groups reached percentage of beating EBs of above 95%.

Figure 3.53

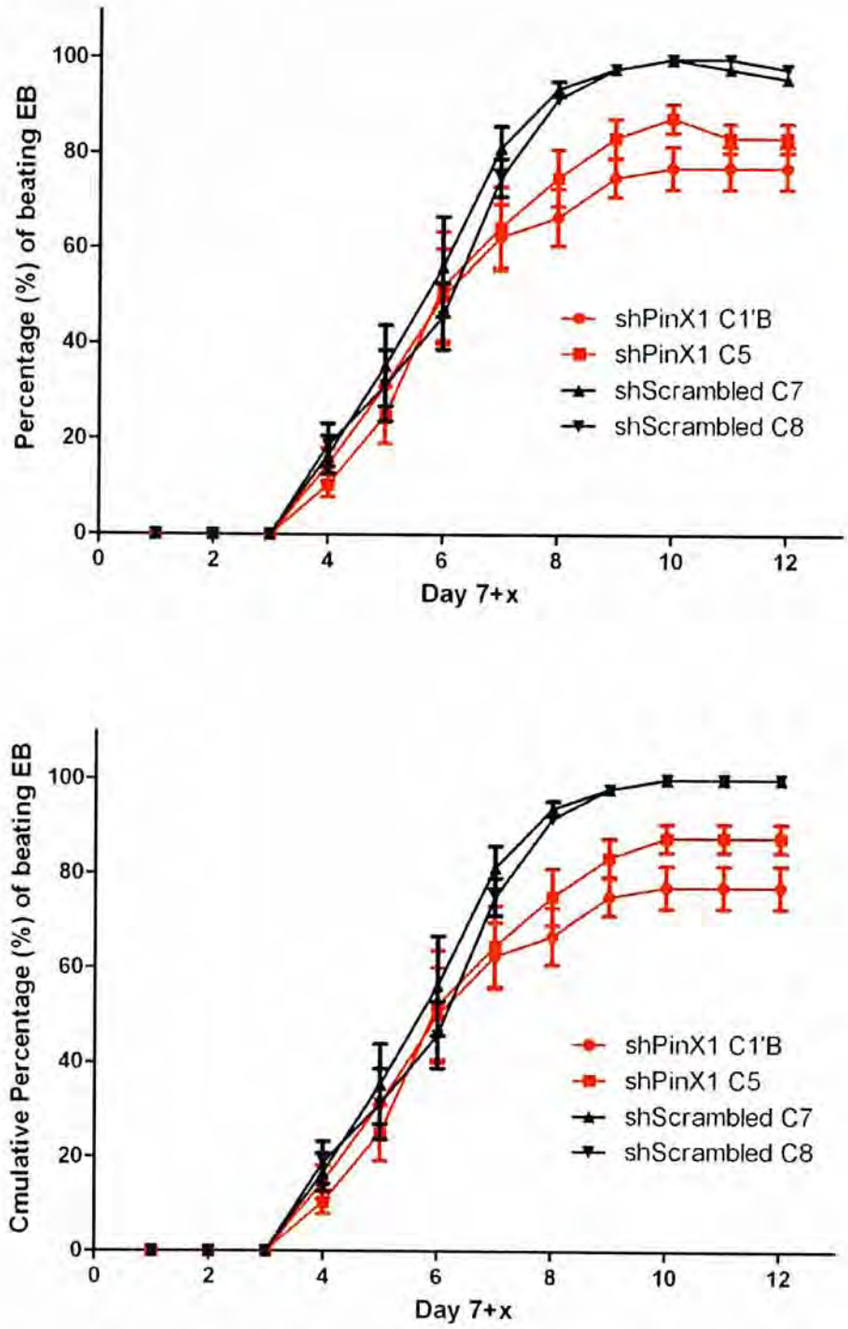


Figure 3.53 (Upper panel) Beating curve and (lower panel) cumulative beating curve of mPinX1 knockdown (shPinX1) stable cell lines and control (shScrambled) cell lines. All the EBs started to beat at around Day 7+4 and reached a maximum percentage of beating at around Day 7+10. For the two clones in shScrambled group, C7 reached a maximum of 98% beating, while C8 reached a maximum of 100% beating. For the two clones in shPinX1 group, C1'B reached a maximum of 77% beating, while C8 reached a maximum of 87% beating. Knockdown of mPinX1 showed a trend of decrease in the maximum percentage of beating EBs formed.

CHAPTER FOUR DISCUSSIONS

Embryonic stem cells are known to have a high telomerase activity. This high activity is involved in sustaining the indefinite proliferation ability [8, 56], differentiation process [6] and stress resistance [48]. On the other hand, previous studies showed that human PinX1 is a telomerase inhibitor [112]. In this project, the effect of mPinX1, and its novel splice variant mPinX1t, on the characteristics of mESCs were studied. Transient and stable over-expression and knockdown of mPinX1 and mPinX1t were performed in mESCs; their influences on the telomerase activity, mESC proliferation, viability, cell cycle progression, pluripotency and differentiation ability were examined.

4.1 mPinX1t gene was detected in mESCs

mPinX1t transcript, which is a possible splice variant of mPinX1, was detected in mESCs and their differentiation derivatives. It contains the mPinX1 gene sequence with exception of the additional 111 nucleotides in between two mPinX1 exons. The 111 additional nucleotides was found in Locus NT_039606.7 in the mouse genome where the PinX1 gene was coded for. Since the template used for the PCR experiment was made by reverse transcription using Poly-T as primer, it is very likely that the mPinX1t transcript is a functional mRNA with poly-A tail. Since a

stop codon is present in the additional sequence, the predicted polypeptide chain encoded by mPinX1t transcript is consisted of the N-terminal but not the C-terminal of mPinX1. Consistently, western blot result showed that the exogenously expressed His-mPinX1t gene encodes for a polypeptide with a size of around 30-35 kDa; given the His tag is of a predicted size of 8kDa, the mPinX1t is therefore of a size of around 22-27 kDa, which is around half of the size of mPinX1 protein (47 kDa). Since many splice variants were shown to be involved in the regulation of the gene or in their related functions, it would be necessary also to find out if mPinX1t would regulate mPinX1 gene or whether it would also regulate the telomerase activity in mESCs. Besides, mPinX1t would provide a good opportunity to understand the cellular function of the N-terminal of mPinX1. Northern blot analysis would be done in the future to further confirm the presence of the full transcript of mPinX1t in mESCs.

4.2 Presence of mPinX1 and mPinX1t in mouse tissues, mESCs and their differentiation derivatives

hPinX1 expression was found to be decreased in a variety of human cancers [41, 51]. PinX1 heterozygous knockout mice also showed an increased frequency of developing tumor [111]. In this study, routine PCR and RT-qPCR results showed that

both mPinX1 and mPinX1t gene were expressed in various mouse tissues, mESCs and their differentiation derivatives. This is in line with the studies of other PinX1 homologs, which were found to be ubiquitously expressed in various normal tissues in different species [51, 52, 68, 84]. Since the expression of telomerase in normal somatic cells is very low or not detectable, the presence of PinX1 in normal tissues hints that PinX1 may be essential for maintaining other cellular functions. Previous studies have shown that yeast PinX1 is crucial for pre-ribosomal RNA processing and small nucleolar RNA maturation [36]. In addition, hPinX1 was also found to be involved in chromosome segregation and congression during mitosis [50, 108], yet the exact role of PinX1 in somatic cells still awaits to be explored.

4.3 Differences in expression of mPinX1 and mPinX1t in undifferentiated mESCs and their differentiation derivatives

RT-qPCR results showed that the expression of mPinX1 was higher in undifferentiated mESCs than their differentiation derivatives, while for mPinX1t, the expression pattern was opposite. Western blot results also demonstrated a consistent change of mPinX1 protein expression during differentiation. However, the endogenous mPinX1t protein could not be detected in both undifferentiated mESCs and its differentiation derivatives, which might due to the low expression level, as its

mRNA expression was much lower (around 10-20 fold) than that of mPinX1 gene. Switching expression level of splice variants of some genes was found to associate with stem cell differentiation and pluripotency [77, 105]; it is speculated that a change in expression of mPinX1 and its splice variant mPinX1t might also take part in these processes in mESCs. A decrease of expression of mPinX1 and increase of expression of mPinX1t as mESCs differentiate hint that the splice variants might be involved in the differentiation process of mESCs.

Many studies have shown that telomerase activity decreased during differentiation, this might be due to the fact that differentiation causes the cells to exit from the cell cycle; since telomerase activity is positively related to the proliferation status, differentiated cells which have a lower proliferative capacity would have a lower telomerase activity. On the other hand, mechanisms controlling differentiation may down-regulate telomerase activity [6, 74, 76]. As a telomerase regulator, expression of mPinX1/ mPinX1t might change during differentiation in accordance to the down-regulation of the telomerase activity. Alternatively, the change in expression of mPinX1/mPinX1t could also be attributed to their involvement in differentiation [85]. A recent finding showed that during *Xenopus* embryogenesis, expression level of TRF1 and PinX1 mRNA were constant at stages 8 to 10 and then decreased afterwards, suggesting that PinX1 might be involved in embryonic development [91].

Consistently, the mRNA expression of mPinX1 was also shown to decrease as differentiation occurs. Moreover, as telomere length is known to decrease upon differentiation [109], it is also imaginable that the need and hence expression level of TRF1 (being a telomeric protein), and PinX1 (being an interacting protein of TRF1) decrease together with telomere length upon differentiation.

From the western blot results, the band located at around 70kDa is possibly a post-translationally modified form of mPinX1, as it also disappeared upon peptide pre-incubation and the expected size of mPinX1 is around 47kDa. This modified form was up-regulated upon differentiation, hinting that post-translation modification of mPinX1 might be associated with differentiation process of mESCs. In the future, we would investigate this possibility by identifying the type of post-translational modification, followed by blocking this modification to see if there is any effect on the differentiation of mESCs.

4.4 mPinX1 and mPinX1t are pre-dominantly localized in the nucleolus

mPinX1 pre-dominantly localized in the nucleolus, with some located inside the nucleus, similar to the location of other homologs [16, 36, 68, 84]. Being in the nucleolus, mPinX1 might be involved in some RNA processing activities. mPinX1 possesses the G-patch domain at the N-terminus, which is known to be conserved for

RNA processing proteins in eukaryotes [5]. Indeed, the yeast homolog of PinX1 was shown to be involved in rRNA processing and small nucleolar RNA maturation [36]. Besides, it is possible for mPinX1 to interact with TERT inside the nucleolus, as TERT was found to undergo a regulated translocation between the nucleoplasm and nucleolus [100]. The mPinX1 localized inside the nucleoplasm might interact with the functioning telomerase and TRF1, as they are both located inside the nucleoplasm.

mPinX1t possesses only the N-terminal, but not the C-terminal domain, of mPinX1. Since both mPinX1 and mPinX1t localized mainly inside the nucleolus, this hints that the nucleolar signaling domain or structure resides in the N-terminal domain of mPinX1. In fact previous studies showed various contrasting results of the region in PinX1 sequence on protein localization. In one study, hPinX1 C-terminal deletion mutant inhibited the protein nucleolar localization (using an HA tag at N-terminus) [107], while in another study on hPinX1, deletion on N-terminal or C-terminal did not alter its nucleolar localization (using RFP tag at C-terminus) [54]. Deletion of N-terminus in rPinX1 was found to inhibit its localization inside nucleolus (using HA tag at N-terminus) [68]. It is currently not clear that why many different results were found from other groups, but it is possible that the different location of tags might cause the difference.

4.5 mPinX1 and mPinX1t interacted with mTERT

PinX1 was shown to bind to telomerase subunit TERT and inhibit telomerase activities in several species [54, 84, 112]. Both N-terminal and C-terminal of hPinX1 could interact with hTERT [16, 112]. Our data showed that mPinX1 interacted with mTERT *in vivo*. mPinX1t, which lacked the C-terminal, also interacted with mTERT, indicating that the N-terminal alone of mPinX1 is sufficient for its interaction with mTERT. Previous studies on hPinX1 showed that both the N-terminus and C-terminus of hPinX1 interact with hTERT, but only the C-terminus was found to be important for inhibiting telomerase activity. Moreover, there was no study showing how the interaction directly contributes to its negative regulation on telomerase activity. Thus, the function of this interaction between PinX1 and TERT remains to be elucidated.

4.6 Transient knockdown of mPinX1 slightly inhibited, while over-expression of mPinX1 slightly promoted telomerase activity

Previous studies have shown that PinX1 is a negative regulator of telomerase in cancer cells [93, 110, 112] and normal somatic cells [84, 111]. In our experiments, transient knockdown of mPinX1 in mESCs slightly inhibited telomerase activity (around 15%), while over-expression of mPinX1 in mESCs slightly promoted

telomerase activity (around 10%). These results suggested that mPinX1 may not act as a telomerase inhibitor in mESCs as in other cell lines; on the contrary, it promotes telomerase activity.

There is so far no study showing the positive role of mPinX1 on telomerase activity. Knock-down of mPinX1 in mice increased the frequency of tumor formation; down-regulation of PinX1 was found in many cancer cells [13, 58]. Its down-regulation might contribute to telomerase activation in cancer cells, but this mechanism might not be necessary in mESCs as mPinX1 expression level is relatively high in mESCs (when compared to that of differentiated cells). Although both ESCs and cancer cells has a relatively high telomerase activity, PinX1 expresses robustly in ESCs while its expression is downregulated/dysregulated in cancer cells. This difference in expression of mPinX1 hints that the function of PinX1 may be different in ESCs and cancer cells, the up-regulated telomerase activity caused by decreased expression of PinX1 in cancer cells may not apply in ESCs. Besides, although mPinX1 was found to interact with mTERT in our experiments, no study has been done on how this interaction directly contributes to its negative regulation on telomerase activity. It is also possible that mPinX1 would affect telomerase activity, for example, by acting at the post-transcriptional level of mTERT transcript (as mPinX1 contains a RNA binding G-patch), or by interacting with telomerase

associated proteins. PinX1 was found to be an interacting partner of the telomeric protein TRF1. However, previous study showed that disrupting the interaction between PinX1 and TRF1 would not affect its telomerase regulation activity [82]. It is possible that mPinX1 would have other interacting partners in mESCs which are at present unknown. These interactions might influence whether PinX1 would act as an inhibitor or activator of telomerase activity. In the future, we could investigate this possibility by identifying the interacting partners of mPinX1 in mESCs and comparing with that of cancer cells.

In mESCs, a high level of telomerase activity is needed to maintain the self-renewal state by giving mESCs the ability to proliferate indefinitely with a stable telomere length [8, 56]. Besides, telomerase activity is crucial in differentiation processes [6] and stress resistance [48]. Studies have shown that the major mechanism of regulation of telomerase activity is the transcriptional regulation of the TERT gene [1]. In ESCs, there are many transcription factors, including the c-Myc, Hif1-α, Klf-4, SP1 and STAT3, which are robustly expressed to maintain the high level of expression of TERT [1, 22, 42, 94, 99, 101]. In this cellular context, the regulation of telomerase activity by mPinX1 would be dispensable, so mPinX1 might not be the rate-limiting physiological regulator of telomerase in mESCs, as shown by the minor change in telomerase activity caused by the alteration of mPinX1 expression.

In the future, another TRAP assay utilizing a gel based detection method by radioactive or non-radioactive means would be used to further confirm the change in telomerase activity we saw in these experiments [110]. Also, TRAP assay could be done on the differentiation derivatives of stable mPinX1 over-expression and knockdown mESCs to compare the effect of mPinX1 on telomerase in both undifferentiated mESCs and its differentiation derivatives.

4.7 Both transient knockdown and over-expression of mPinX1 inhibited the growth of mESCs

Both cell cycle progression and pluripotency will affect the proliferation of mESCs. For the former one, if the gene promotes or inhibits transition of cell cycle phases, cell proliferation rate would be increased or decreased, respectively. Loss of pluripotency will cause the cells to leave the cell cycle and start differentiation, thus decreasing the rate of cell proliferation. However, our results showed that knockdown or over-expression of mPinX1 did not affect both the cell cycle distribution and the pluripotency. It is possible that changing the expression of mPinX1 affects some processes which do not specifically alter the transition of specific cell cycle phases, but lengthen each phase of the cell cycle; this could also lead to a decrease in cell proliferation.

Besides telomerase regulation, PinX1 was found to be recruited in many cellular activities. PinX1 was shown to involve in chromosome congression and segregation by binding to microtubules and nucleolin [50, 108]. On the other hand, PinX1 was also found to be crucial for pre-ribosomal RNA processing and small nucleolar RNA maturation [36]. Knockdown of mPinX1 might affect these cellular activities in addition to affecting the telomerase activity; this would result in a decrease in cell proliferation.

Although many papers related the growth inhibition of over-expressing PinX1 to its effect on inhibiting telomerase activity, the involvement of other related pathways causing crisis cannot be excluded. Over-expression of mPinX1 might also affect other pathways which also contribute to growth inhibition. Moreover, PinX1 was found to be recruited by TRF1 to inhibit telomerase binding to telomere [82], so over-expression of mPinX1 might increase the PinX1-TRF1 complexes on telomere which prevent telomerase interacting with telomere.

In the future, to investigate if there is telomerase-independent effect of mPinX1 in mESCs, we can use TERT knockout cell lines to study the downstream effect of mPinX1 on other cellular processes which might affect cell proliferation.

4.8 Both stable knockdown and over-expression of mPinX1 did not affect cell proliferation and telomerase activity of mESCs

Diverged from what we found in transient knockdown and over-expression experiments, both stable knockdown and over-expression did not exert significant effect on cell proliferation and telomerase activity. It was quite surprising because we anticipated that the effect in transient experiments could be magnified in stable cell lines. Throughout the experiment, GFP signals from all groups were observed under fluorescence microscope frequently to make sure that the genes/ shRNA were robustly expressing. As discussed before, there are many other pathways in mESCs regulating telomerase activity, mPinX1 might not be the rate-limiting physiological regulator of telomerase and so its effect on telomerase activity would be very minor. These telomerase regulating pathways might co-ordinate in a way that the slight changes in telomerase activity caused by the alteration in mPinX1 expression would be diminished for the cell to come back to the original homeostatic state; this can explain why cell proliferation was not affected as well. These compensatory mechanisms might need a longer time to respond to the change, so the effect could be seen in transient experiments but not in stable cell lines.

4.9 Involvement of mPinX1 and mPinX1t in the differentiation process of mESCs

From the qPCR results showing the significant decrease of expression of mPinX1 during differentiation, it is very possible that its main function is on the differentiation process. Preliminary results of differentiation of the stable cell lines showed that over-expression or knockdown of mPinX1 did not lead to a significant change in the size of EBs formed at day 7. However, over-expression of mPinX1 advanced the time of reaching maximum percentage of beating EBs, while knockdown of mPinX1 decreased the percentage of beating EBs formed. These results suggested that mPinX1 is essential for cardiac differentiation. However, it is at present indistinguishable whether the effect of mPinX1 is on general mesodermal differentiation, or specifically on cardiogenic differentiation. Besides, as the size of the beating EBs could vary a lot, the percentage of cardiomyocytes present in the population could not be fully reflected by the number of beating clusters. Therefore, in the future, quantitative measurement of the expression of 1) three germ layer makers (e.g. ectoderm: Sox1; mesoderm: Mixl1, Brachyury T, Nodal; endoderm: HNF-1β) on early differentiation days; 2) cardiac specific transcription factors (e.g. GATA-4 and Nkx2.5) on early differentiation days; and 3) cardiac structural protein genes (e.g. Mlc2a, Mlc2v, cardiac actin, α-MHC, troponin T) on late differentiation

days would be done to evaluate the mechanism behind. In addition, more independent experiments (n numbers) have to be conducted to obtain more convincing data.

Up to date, no study has been conducted about the effect of PinX1 on the ESC differentiation process, but a recent paper showed that total knockout of PinX1 is embryonic lethal, while some of the PinX1 heterozygous embryos died in *utero*, indicating PinX1 is essential for embryonic development [111]. Besides, it was found that over-expressing telomerase activity in murine ESCs enhanced differentiation towards the hematopoietic lineage [6]. In fact, enhanced hematopoietic differentiation was found to impair cardiac differentiation and *vice versa*. For example, ESCs with $Wnt2^{-/-}$ was found to have impaired terminal cardiac differentiation, but enhanced hematopoietic cell differentiation [92]. Also Wnt-1 over-expression enforced cardiac differentiation, but inhibited hematopoietic cell differentiation in ESCs [97]. Taken together, mPinX1 is involved in the differentiation process, and it is possible that mPinX1 would regulate telomerase activity during differentiation of ESCs, thereby affecting the differentiation pattern in mesoderm layer.

Therefore, in the future, quantitative measurement on the genes of other lineages, including the hematopoietic lineage, will be done to see if mPinX1 would also affect

the differentiation of other lineages. TRAP assay could be done to investigate if mPinX1 would affect telomerase activity during differentiation.

Over-expression of mPinX1t caused a slight delay of the onset of spontaneous beating in EBs. From qPCR data, its expression decreased during differentiation, in a way opposite to the expression pattern of mPinX1. It is possible that over-expression of mPinX1t would exert a different effect on differentiation when compared with the effect seen with over-expressing mPinX1. The experiment would need to be repeated in the future to get more convincing data.

4.10 Regulation of mPinX1 gene expression by mPinX1t

In both transient and stable over-expression of mPinX1t, the expression of mPinX1 gene was elevated. However, over-expression of mPinX1 did not alter the expression of mPinX1t. This hints that mPinX1t might be involved in positively regulating the level of mPinX1. mPinX1t gene consists of the N-terminal of mPinX1, which contains a G-patch region that could bind RNA, together with the additional peptide sequence (which is present in mPinX1t but not mPinX1), mPinX1t might bind to the mRNA of mPinX1 and post-transcriptionally regulate it. Although mPinX1 protein also possesses the same N-terminal G-patch region, the secondary structure of the C-terminus of mPinX1 might mask its own G-patch region, thus only mPinX1t

protein (which does not contain the C-terminus of mPinX1) could bind to the mPinX1 mRNA and regulate it while mPinX1 protein could not. Many genes were shown to be regulated by their own splice variants [47, 65]. Over-expression of mPinX1t did not affect telomerase activity, cell cycle progression and pluripotency, suggesting that it is also not involved in these cellular processes. The decrease in cell proliferation in transient over-expression of mPinX1t might due to the effect of increased mPinX1 expression.

It would be more convincing if we can see the opposite effect with the knockdown of mPinX1t, however, the siRNAs against mPinX1t failed to work with unknown reasons. In the future, we could investigate whether mPinX1t would bind to mPinX1 mRNA to regulate mPinX1 gene expression.

4.11 Future perspectives

In the present study, the role of mPinX1 on telomerase activity and other cellular processes in mESCs were investigated. It was found that mPinX1 slightly promoted telomerase activity in mESCs in transient knockdown and over-expression experiments but not in stable cell lines. It would be more convincing to detect the telomerase activity again using another TRAP assay in these experiments. Both transient over-expression and knockdown of gene was found to inhibit proliferation.

It would be interesting to know the other pathways that mPinX1 would be involved in for cell proliferation. Therefore, TERT knockout mESCs cell lines would be used in the future to study the functions of mPinX1. In long term over-expression and knockdown of mPinX1 and mPinX1t, more independent experiments would be added to the cell cycle analysis and western blot analysis on pluripotent markers.

Results also showed that mPinX1 would affect the differentiation pattern of mESCs. Whether this effect is caused by alteration in telomerase activity by mPinX1 during differentiation, and whether the differentiation lineages would be affected by mPinX1, would be studied in the future by quantitative measurements of the respective genes involved and TRAP assay. More n will also be added to make the data more convincing.

Besides, many puzzles about the function of the splice variant mPinX1t are awaited to be solved. The presence of the full transcript of mPinX1t in mESCs would be confirmed by northern blot analysis. Its function on mPinX1 gene regulation would be investigated in the future by looking at its interaction with mPinX1 mRNA. In addition, experiments would be conducted to study its effect on differentiation of mESCs.

CHAPTER FIVE CONCLUSION

In summary, a telomerase regulator mPinX1 and its novel splice variant mPinX1t were characterized and their roles in maintaining mESCs characteristics were studied. Both mPinX1 and mPinX1t were found to be present in all investigated mouse tissues, undifferentiated mESCs and their Day 7+25 differentiation derivatives. The expression of mPinX1 was down-regulated upon differentiation while for mPinX1t the expression pattern was opposite. Both of them were found to be pre-dominantly localized in the nucleolus of mESCs, and they were found to interact with mTERT *in vivo*.

To study their roles in maintaining mESCs characteristics, transient knockdown of mPinX1, and over-expression of mPinX1 and mPinX1t were done in mESCs. mPinX1 knockdown slightly decreased telomerase activity, while over-expression slightly increased telomerase activity, suggesting that PinX1 did not act as a telomerase inhibitor in mESCs at cellular level. Both transient knockdown and over-expression of mPinX1 caused an inhibitory effect on cell proliferation without affecting cell cycle distribution and pluripotency. Over-expression of mPinX1t up-regulated mPinX1 mRNA expression, suggesting its regulatory role on mPinX1 gene expression. Transient over-expression of mPinX1t also caused an inhibitory

effect on cell proliferation without affecting cell cycle distribution and pluripotency, which might be due to the effect of increased mPinX1 expression. Further experiments are needed to verify its role on mPinX1 regulation.

Diverged from the results of transient experiments, stable knockdown and over-expression of mPinX1 did not cause any significant effect on cell proliferation and telomerase activity. These stable cell lines were subjected to differentiation. Preliminary results showed that the size of EBs formed from all groups had no difference at Day 7. On the other hand, mPinX1 over-expressed groups caused an advanced onset of time in reaching maximum beating EBs, while mPinX1 knockdown groups caused a decreased percentage of beating EBs formed. Over-expression of mPinX1t also caused a slight delay of onset of spontaneous beating EBs, suggesting a possible role of mPinX1 and mPinX1t in mESCs differentiation.

CHAPTER SIX REFERENCES

1. Aisner, D.L., W.E. Wright, and J.W. Shay, *Telomerase regulation: not just flipping the switch.* Curr Opin Genet Dev, 2002. **12**(1): p. 80-5.
2. Albanell, J., et al., *Telomerase activity is repressed during differentiation of maturation-sensitive but not resistant human tumor cell lines.* Cancer Res, 1996. **56**(7): p. 1503-8.
3. Allsopp, R.C., et al., *Telomere length predicts replicative capacity of human fibroblasts.* Proc Natl Acad Sci U S A, 1992. **89**(21): p. 10114-8.
4. Ancelin, K., et al., *Targeting assay to study the cis functions of human telomeric proteins: evidence for inhibition of telomerase by TRF1 and for activation of telomere degradation by TRF2.* Mol Cell Biol, 2002. **22**(10): p. 3474-87.
5. Aravind, L. and E.V. Koonin, *G-patch: a new conserved domain in eukaryotic RNA-processing proteins and type D retroviral polyproteins.* Trends Biochem Sci, 1999. **24**(9): p. 342-4.
6. Armstrong, L., et al., *Overexpression of telomerase confers growth advantage, stress resistance, and enhanced differentiation of ESCs toward the hematopoietic lineage.* Stem Cells, 2005. **23**(4): p. 516-29.
7. Assady, S., et al., *Insulin production by human embryonic stem cells.* Diabetes, 2001. **50**(8): p. 1691-7.
8. Blasco, M.A., *Telomeres and human disease: ageing, cancer and beyond.* Nat Rev Genet, 2005. **6**(8): p. 611-22.
9. Bodnar, A.G., et al., *Extension of life-span by introduction of telomerase into normal human cells.* Science, 1998. **279**(5349): p. 349-52.
10. Bonatz, G., et al., *High telomerase activity is associated with cell cycle deregulation and rapid progression in endometrioid adenocarcinoma of the uterus.* Hum Pathol, 2001. **32**(6): p. 605-14.
11. Burdon, T., A. Smith, and P. Savatier, *Signalling, cell cycle and pluripotency in embryonic stem cells.* Trends Cell Biol, 2002. **12**(9): p. 432-8.
12. Cabrera, C.M., et al., *Strategies for preventing immunologic rejection of transplanted human embryonic stem cells.* Cytotherapy, 2006. **8**(5): p. 517-8.
13. Cai, M.Y., et al., *Decreased expression of PinX1 protein is correlated with tumor development and is a new independent poor prognostic factor in ovarian carcinoma.* Cancer Sci. **101**(6): p. 1543-9.
14. Celli, G.B. and T. de Lange, *DNA processing is not required for ATM-mediated telomere damage response after TRF2 deletion.* Nat Cell Biol, 2005. **7**(7): p. 712-8.
15. Cesare, A.J., et al., *Telomere looping in P. sativum (common garden pea).*

Plant J, 2003. **36**(2): p. 271-9.

16. Chen, G., et al., *C-terminal amino acids 290-328 of LPTS/PinX1 confer telomerase inhibition.* Biochem Biophys Res Commun. **398**(4): p. 683-9.
17. Choudhury, A.R., et al., *Cdkn1a deletion improves stem cell function and lifespan of mice with dysfunctional telomeres without accelerating cancer formation.* Nat Genet, 2007. **39**(1): p. 99-105.
18. Collins, K., *Physiological assembly and activity of human telomerase complexes.* Mech Ageing Dev, 2008. **129**(1-2): p. 91-8.
19. Cong, Y.S., J. Wen, and S. Bacchetti, *The human telomerase catalytic subunit hTERT: organization of the gene and characterization of the promoter.* Hum Mol Genet, 1999. **8**(1): p. 137-42.
20. Counter, C.M., et al., *Telomerase activity in normal leukocytes and in hematologic malignancies.* Blood, 1995. **85**(9): p. 2315-20.
21. Counter, C.M., et al., *Telomerase activity is restored in human cells by ectopic expression of hTERT (hEST2), the catalytic subunit of telomerase.* Oncogene, 1998. **16**(9): p. 1217-22.
22. Coussens, M., et al., *RNAi screen for telomerase reverse transcriptase transcriptional regulators identifies HIF1alpha as critical for telomerase function in murine embryonic stem cells.* Proc Natl Acad Sci U S A. **107**(31): p. 13842-7.
23. Crowe, D.L., et al., *E2F-1 represses transcription of the human telomerase reverse transcriptase gene.* Nucleic Acids Res, 2001. **29**(13): p. 2789-94.
24. d'Adda di Fagagna, F., et al., *A DNA damage checkpoint response in telomere-initiated senescence.* Nature, 2003. **426**(6963): p. 194-8.
25. Darr, H., Y. Mayshar, and N. Benvenisty, *Overexpression of NANOG in human ES cells enables feeder-free growth while inducing primitive ectoderm features.* Development, 2006. **133**(6): p. 1193-201.
26. de Lange, T., *How Shelterin Solves the Telomere End-Protection Problem.* Cold Spring Harb Symp Quant Biol.
27. de Lange, T., *Shelterin: the protein complex that shapes and safeguards human telomeres.* Genes Dev, 2005. **19**(18): p. 2100-10.
28. de Lange, T., *How telomeres solve the end-protection problem.* Science, 2009. **326**(5955): p. 948-52.
29. Denchi, E.L., *Give me a break: how telomeres suppress the DNA damage response.* DNA Repair (Amst), 2009. **8**(9): p. 1118-26.
30. Ebert, A.D. and C.N. Svendsen, *Human stem cells and drug screening: opportunities and challenges.* Nat Rev Drug Discov. **9**(5): p. 367-72.
31. Elenitoba-Johnson, K.S., *Complex regulation of telomerase activity: implications for cancer therapy.* Am J Pathol, 2001. **159**(2): p. 405-10.

32. Evans, M.J. and M.H. Kaufman, *Establishment in culture of pluripotential cells from mouse embryos.* Nature, 1981. **292**(5819): p. 154-6.
33. Feldser, D.M. and C.W. Greider, *Short telomeres limit tumor progression in vivo by inducing senescence.* Cancer Cell, 2007. **11**(5): p. 461-9.
34. Feng, J., et al., *The RNA component of human telomerase.* Science, 1995. **269**(5228): p. 1236-41.
35. Flores, I., M.L. Cayuela, and M.A. Blasco, *Effects of telomerase and telomere length on epidermal stem cell behavior.* Science, 2005. **309**(5738): p. 1253-6.
36. Guglielmi, B. and M. Werner, *The yeast homolog of human PinX1 is involved in rRNA and small nucleolar RNA maturation, not in telomere elongation inhibition.* J Biol Chem, 2002. **277**(38): p. 35712-9.
37. Guo, X. and R.S. Hartley, *HuR contributes to cyclin E1 deregulation in MCF-7 breast cancer cells.* Cancer Res, 2006. **66**(16): p. 7948-56.
38. Hodgson, D.M., et al., *Stable benefit of embryonic stem cell therapy in myocardial infarction.* Am J Physiol Heart Circ Physiol, 2004. **287**(2): p. H471-9.
39. Jiang, X.R., et al., *Telomerase expression in human somatic cells does not induce changes associated with a transformed phenotype.* Nat Genet, 1999. **21**(1): p. 111-4.
40. Karlseder, J., et al., *p53- and ATM-dependent apoptosis induced by telomeres lacking TRF2.* Science, 1999. **283**(5406): p. 1321-5.
41. Kondo, T., et al., *Loss of heterozygosity and histone hypoacetylation of the PINX1 gene are associated with reduced expression in gastric carcinoma.* Oncogene, 2005. **24**(1): p. 157-64.
42. Konnikova, L., et al., *Signal transducer and activator of transcription 3 (STAT3) regulates human telomerase reverse transcriptase (hTERT) expression in human cancer and primary cells.* Cancer Res, 2005. **65**(15): p. 6516-20.
43. Krek, W. and E.A. Nigg, *Mutations of p34cdc2 phosphorylation sites induce premature mitotic events in HeLa cells: evidence for a double block to p34cdc2 kinase activation in vertebrates.* Embo J, 1991. **10**(11): p. 3331-41.
44. Kruk, P.A., et al., *Telomere reduction and telomerase inactivation during neuronal cell differentiation.* Biochem Biophys Res Commun, 1996. **224**(2): p. 487-92.
45. Kyo, S., et al., *Sp1 cooperates with c-Myc to activate transcription of the human telomerase reverse transcriptase gene (hTERT).* Nucleic Acids Res, 2000. **28**(3): p. 669-77.
46. Landberg, G., et al., *Telomerase activity is associated with cell cycle deregulation in human breast cancer.* Cancer Res, 1997. **57**(3): p. 549-54.

47. Lareau, L.F., et al., *The evolving roles of alternative splicing.* Curr Opin Struct Biol, 2004. **14**(3): p. 273-82.
48. Lee, M.K., M.P. Hande, and K. Sabapathy, *Ectopic mTERT expression in mouse embryonic stem cells does not affect differentiation but confers resistance to differentiation- and stress-induced p53-dependent apoptosis.* J Cell Sci, 2005. **118**(Pt 4): p. 819-29.
49. Letso, R.R. and B.R. Stockwell, *Chemical biology: renewing embryonic stem cells.* Nature, 2006. **444**(7120): p. 692-3.
50. Li, N., et al., *PinX1 is recruited to the mitotic chromosome periphery by Nucleolin and facilitates chromosome congression.* Biochem Biophys Res Commun, 2009. **384**(1): p. 76-81.
51. Liao, C., et al., *Identification of the gene for a novel liver-related putative tumor suppressor at a high-frequency loss of heterozygosity region of chromosome 8p23 in human hepatocellular carcinoma.* Hepatology, 2000. **32**(4 Pt 1): p. 721-7.
52. Liao, C., M.J. Zhao, and Z.P. Li, *[The cloning and expression of a novel mouse gene mLPTS and its subcellular localization].* Yi Chuan Xue Bao, 2002. **29**(10): p. 865-70.
53. Lin, J. and E.H. Blackburn, *Nucleolar protein PinX1p regulates telomerase by sequestering its protein catalytic subunit in an inactive complex lacking telomerase RNA.* Genes Dev, 2004. **18**(4): p. 387-96.
54. Lin, J., et al., *Characterization of a novel effect of hPinX1 on hTERT nucleolar localization.* Biochem Biophys Res Commun, 2007. **353**(4): p. 946-52.
55. Lindvall, O. and R. McKay, *Brain repair by cell replacement and regeneration.* Proc Natl Acad Sci U S A, 2003. **100**(13): p. 7430-1.
56. Liu, L., et al., *An essential role for functional telomeres in mouse germ cells during fertilization and early development.* Dev Biol, 2002. **249**(1): p. 74-84.
57. Liu, L., et al., *Telomerase deficiency impairs differentiation of mesenchymal stem cells.* Exp Cell Res, 2004. **294**(1): p. 1-8.
58. Ma, Y., et al., *The correlation of genetic instability of PINX1 gene to clinico-pathological features of gastric cancer in the Chinese population.* J Cancer Res Clin Oncol, 2009. **135**(3): p. 431-7.
59. Martin, G.R., *Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells.* Proc Natl Acad Sci U S A, 1981. **78**(12): p. 7634-8.
60. Martinez, P., et al., *Mammalian Rap1 controls telomere function and gene expression through binding to telomeric and extratelomeric sites.* Nat Cell Biol. **12**(8): p. 768-80.

61. Maser, R.S. and R.A. DePinho, *Connecting chromosomes, crisis, and cancer.* Science, 2002. **297**(5581): p. 565-9.
62. Misiti, S., et al., *Induction of hTERT expression and telomerase activity by estrogens in human ovary epithelium cells.* Mol Cell Biol, 2000. **20**(11): p. 3764-71.
63. Munoz-Jordan, J.L., et al., *t-loops at trypanosome telomeres.* Embo J, 2001. **20**(3): p. 579-88.
64. Nakamura, T.M., et al., *Telomerase catalytic subunit homologs from fission yeast and human.* Science, 1997. **277**(5328): p. 955-9.
65. Ni, J.Z., et al., *Ultraconserved elements are associated with homeostatic control of splicing regulators by alternative splicing and nonsense-mediated decay.* Genes Dev, 2007. **21**(6): p. 708-18.
66. O'Connor, M.S., et al., *A critical role for TPP1 and TIN2 interaction in high-order telomeric complex assembly.* Proc Natl Acad Sci U S A, 2006. **103**(32): p. 11874-9.
67. Odorico, J.S., D.S. Kaufman, and J.A. Thomson, *Multilineage differentiation from human embryonic stem cell lines.* Stem Cells, 2001. **19**(3): p. 193-204.
68. Oh, B.K., et al., *Rat homolog of PinX1 is a nucleolar protein involved in the regulation of telomere length.* Gene, 2007. **400**(1-2): p. 35-43.
69. Oh, S., et al., *Identification of Mad as a repressor of the human telomerase (hTERT) gene.* Oncogene, 2000. **19**(11): p. 1485-90.
70. Okita, K. and S. Yamanaka, *Intracellular signaling pathways regulating pluripotency of embryonic stem cells.* Curr Stem Cell Res Ther, 2006. **1**(1): p. 103-11.
71. Pendino, F., et al., *Retinoids down-regulate telomerase and telomere length in a pathway distinct from leukemia cell differentiation.* Proc Natl Acad Sci U S A, 2001. **98**(12): p. 6662-7.
72. Prowse, K.R. and C.W. Greider, *Developmental and tissue-specific regulation of mouse telomerase and telomere length.* Proc Natl Acad Sci U S A, 1995. **92**(11): p. 4818-22.
73. Resnitzky, D., et al., *Acceleration of the G1/S phase transition by expression of cyclins D1 and E with an inducible system.* Mol Cell Biol, 1994. **14**(3): p. 1669-79.
74. Richardson, R.M., et al., *Ectopic telomerase expression inhibits neuronal differentiation of NT2 neural progenitor cells.* Neurosci Lett, 2007. **421**(2): p. 168-72.
75. Rodda, D.J., et al., *Transcriptional regulation of nanog by OCT4 and SOX2.* J Biol Chem, 2005. **280**(26): p. 24731-7.
76. Salomonis, N., et al., *Alternative splicing in the differentiation of human*

embryonic stem cells into cardiac precursors. PLoS Comput Biol, 2009. **5**(11): p. e1000553.

77. Salomonis, N., et al., *Alternative splicing regulates mouse embryonic stem cell pluripotency and differentiation.* Proc Natl Acad Sci U S A. **107**(23): p. 10514-9.
78. Savoysky, E., et al., *Down-regulation of telomerase activity is an early event in the differentiation of HL60 cells.* Biochem Biophys Res Commun, 1996. **226**(2): p. 329-34.
79. Sfeir, A., et al., *Loss of Rap1 induces telomere recombination in the absence of NHEJ or a DNA damage signal.* Science. **327**(5973): p. 1657-61.
80. Sharma, H.W., et al., *Differentiation of immortal cells inhibits telomerase activity.* Proc Natl Acad Sci U S A, 1995. **92**(26): p. 12343-6.
81. Shay, J.W. and S. Bacchetti, *A survey of telomerase activity in human cancer.* Eur J Cancer, 1997. **33**(5): p. 787-91.
82. Soohoo, C.Y., et al., *Telomerase inhibitor PinX1 provides a link between TRF1 and telomerase to prevent telomere elongation.* J Biol Chem. **286**(5): p. 3894-906.
83. Stead, E., et al., *Pluripotent cell division cycles are driven by ectopic Cdk2, cyclin A/E and E2F activities.* Oncogene, 2002. **21**(54): p. 8320-33.
84. Sun, C., et al., *Identification of zebrafish LPTS: a gene with similarities to human LPTS/PinX1 that inhibits telomerase activity.* Gene, 2008. **420**(1): p. 90-8.
85. Sun, J., et al., *The expression of telomeric proteins and their probable regulation of telomerase during the differentiation of all-trans-retinoic acid-responsive and -resistant acute promyelocytic leukemia cells.* Int J Hematol, 2005. **82**(3): p. 215-23.
86. Takai, H., A. Smogorzewska, and T. de Lange, *DNA damage foci at dysfunctional telomeres.* Curr Biol, 2003. **13**(17): p. 1549-56.
87. Tanaka, S. and J.F. Diffley, *Deregulated G1-cyclin expression induces genomic instability by preventing efficient pre-RC formation.* Genes Dev, 2002. **16**(20): p. 2639-49.
88. Tejera, A.M., et al., *TPP1 is required for TERT recruitment, telomere elongation during nuclear reprogramming, and normal skin development in mice.* Dev Cell. **18**(5): p. 775-89.
89. Teo, H., et al., *Telomere-independent Rap1 is an IKK adaptor and regulates NF-kappaB-dependent gene expression.* Nat Cell Biol. **12**(8): p. 758-67.
90. van den Heuvel, S., *Cell-cycle regulation.* WormBook, 2005: p. 1-16.
91. Vizlin-Hodzic, D., et al., *Developmental studies of Xenopus shelterin complexes: the message to reset telomere length is already present in the egg.*

Faseb J, 2009. **23**(8): p. 2587-94.

92. Wang, H., et al., *Wnt2 coordinates the commitment of mesoderm to hematopoietic, endothelial, and cardiac lineages in embryoid bodies.* J Biol Chem, 2007. **282**(1): p. 782-91.
93. Wang, H.B., et al., *PinX1 inhibits telomerase activity in gastric cancer cells through Mad1/c-Myc pathway.* J Gastrointest Surg. **14**(8): p. 1227-34.
94. Wang, J., et al., *Myc activates telomerase.* Genes Dev, 1998. **12**(12): p. 1769-74.
95. Wang, R., et al., *Promoter-dependent EGFP expression during embryonic stem cell propagation and differentiation.* Stem Cells Dev, 2008. **17**(2): p. 279-89.
96. Wang, Z., et al., *Progesterone regulates human telomerase reverse transcriptase gene expression via activation of mitogen-activated protein kinase signaling pathway.* Cancer Res, 2000. **60**(19): p. 5376-81.
97. Weisel, K.C., et al., *Wnt1 overexpression leads to enforced cardiomyogenesis and inhibition of hematopoiesis in murine embryonic stem cells.* Stem Cells Dev. **19**(5): p. 745-51.
98. White, J. and S. Dalton, *Cell cycle control of embryonic stem cells.* Stem Cell Rev, 2005. **1**(2): p. 131-8.
99. Wong, C.W., et al., *Kruppel-like transcription factor 4 contributes to maintenance of telomerase activity in stem cells.* Stem Cells. **28**(9): p. 1510-7.
100. Wong, J.M., L. Kusdra, and K. Collins, *Subnuclear shuttling of human telomerase induced by transformation and DNA damage.* Nat Cell Biol, 2002. **4**(9): p. 731-6.
101. Wu, K.J., et al., *Direct activation of TERT transcription by c-MYC.* Nat Genet, 1999. **21**(2): p. 220-4.
102. Xin, H., D. Liu, and Z. Songyang, *The telosome/shelterin complex and its functions.* Genome Biol, 2008. **9**(9): p. 232.
103. Xu, D., et al., *Downregulation of telomerase reverse transcriptase mRNA expression by wild type p53 in human tumor cells.* Oncogene, 2000. **19**(45): p. 5123-33.
104. Yang, J., et al., *Human endothelial cell life extension by telomerase expression.* J Biol Chem, 1999. **274**(37): p. 26141-8.
105. Yang, Z., et al., *Switched alternative splicing of oncogene CoAA during embryonal carcinoma stem cell differentiation.* Nucleic Acids Res, 2007. **35**(6): p. 1919-32.
106. Ying, Q.L., et al., *BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3.* Cell,

2003. **115**(3): p. 281-92.

107. Yoo, J.E., B.K. Oh, and Y.N. Park, *Human PinX1 mediates TRF1 accumulation in nucleolus and enhances TRF1 binding to telomeres.* J Mol Biol, 2009. **388**(5): p. 928-40.

108. Yuan, K., et al., *PinX1 is a novel microtubule-binding protein essential for accurate chromosome segregation.* J Biol Chem, 2009. **284**(34): p. 23072-82.

109. Zeng, X., *Human embryonic stem cells: mechanisms to escape replicative senescence?* Stem Cell Rev, 2007. **3**(4): p. 270-9.

110. Zhang, B., et al., *Silencing PinX1 compromises telomere length maintenance as well as tumorigenicity in telomerase-positive human cancer cells.* Cancer Res, 2009. **69**(1): p. 75-83.

111. Zhou, X.Z., et al., *The telomerase inhibitor PinX1 is a major haploinsufficient tumor suppressor essential for chromosome stability in mice.* J Clin Invest. **121**(4): p. 1266-82.

112. Zhou, X.Z. and K.P. Lu, *The Pin2/TRF1-interacting protein PinX1 is a potent telomerase inhibitor.* Cell, 2001. **107**(3): p. 347-59.